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(54) Title: IMMOBILIZED CYTOKINES

(57) Abstract

The present invention provides immobilized cytokines comprising cytokines bound to a solid, preferably biologically inert, support. The bound cytokine, for example IL-2, retains comparable biological activity when bound to the support. Accordingly, the bound cytokine is able to be utilized repeatedly and/or in significantly smaller quantities, as compared to an individual soluble cytokine. Cytokines of the present invention include, but are not limited to, IL-1-alpha, IL-1 beta, rIL-2, IL-2, IL-3, IL-4, IL-6, MuGMCSF, HuGMCSF, HuGCSF, HuEPO, *alpha*-interferon, *gamma*-interferon, TNF-alpha, HuILGF-I, HuILGF-II, FGFb, TGF-beta-II, HuEGF, HuPDGF.

IMMOBILIZED CYTOKINES

Technical Field

The present invention relates to cytokines that are immobilized on a solid support.

Background of the Invention

A number of biologically active mediators, generally termed cytokines, are produced by a variety of cells. Cytokines are produced on an obligatory basis for the maintenance of normal homeostasis, and also in response to pathological stimuli, such as immunological, infectious, and inflammatory processes. Those cytokines first described as products of lymphocytes are often referred to as "lymphokines," and those cytokines originally described as products of monocytes have been termed "monokines." Certain cytokines are also referred to as growth factors or colony stimulating factors, based on their effect on cell growth.

Examples of cytokines include: the lymphokines interleukin-1 (IL-1), interleukin-2 (IL-2), and interleukin-3 (IL-3); the monokine gamma interferon; and the growth factors granulocyte-macrophage colony stimulating factor (GMCSF) and erythropoietin (EPO).

Various cytokines serve as endogenous regulators (autocrines) and/or as intercellular signals. Many of these cytokines, initially recognized by a single biological activity, have been shown to have multiple, overlapping biological activities, often acting synergistically to amplify the biological response. The ultimate effect on the target cell includes regulation of growth, mobility, differentiation, and/or protein synthesis.

Interleukin-1 (IL-1), also known as lymphocyte activating factor, is produced by human monocytes, lymphocytes, endothelial cells, and fibroblasts. IL-1 promotes lymphocyte differentiation, as indicated by changes in phenotypic cell surface markers. In addition, IL-1 stimulates T-lymphocyte functions and

increases the production of lymphokines such as IL-2, colony-stimulating factors (CSF), B-cell growth factor (BCGF), gamma-interferon (γ-IFN), and lymphocyte-derived chemotactic factors (LDCF), each with their own biological effects. IL-1 also augments the in vitro proliferation, differentiation, and antibody-producing functions of B-lymphocytes. These and other biological activities have made IL-1 a valuable lymphokine in a wide variety of in vivo and in vitro uses.

Interleukin-2 (IL-2) was first termed T-cell growth factor (TCGF) for its ability to induce T-lymphocytes to proliferate and enable normal T-lymphocytes to be maintained continuously in culture. Like IL-1, IL-2 has been found useful in a wide variety of in vivo and in vitro applications. IL-2, when used as a vaccine adjuvant, overcomes genetic nonresponsiveness to malaria sporozoite peptides and enhances protection against Herpes simplex and rabies viruses. See M. F. Good et al., J. Immunol., 141, 972 (1988) and A. Weinberg et al., J. Immunol., 140, 294 (1988).

Among its biological activities when used as a pharmacological agent, in vitro IL-2 results in the proliferation and differentiation of a group of more selective T-cell populations known as lymphokine activated killer cells, tumor infiltrating lymphocytes, and cytotoxic T-cells. Such cells have been demonstrated in vitro to be cytotoxic to allogenic normal target cells and to both immunogenic and nonimmunogenic tumor cells. See S. A. Rosenberg, J. Nat. Can. Inst., 75, 595 (1985); S. A. Rosenberg, J. Immunol., 121, 1951 (1978); and S. A. Rosenberg et al., Science, 233, 1318 (1986).

In vitro lymphokine activated killer cells have been used in combination with the in vivo administration of interleukin-2 to achieve an improved antitumor effect. The infusion of in vitro IL-2 activated killer cells and the concurrent administration of IL-2 has

demonstrated antitumor activity in both animals and humans; such activity generally exceeding that observed with the use of IL-2 or lymphokine activated killer cells individually. See J. J. Mule et al., Science, 225, 1487 (1984); R. Lafrenier, and S. A. Rosenberg, Cancer Res., 45, 3735 (1985); S. A. Rosenberg et al., N. Engl. J. Med., 316, 889 (1987); J. J. Mule et al., J. Immunol., 136, 3899 (1986); H. W. West et al., N. Engl. J. Med., 316, 898 (1987); S. A. Rosenberg et al., N. Engl. J. Med., 313, 1485 (1985).

The growth of tumor infiltrating lymphocytes obtained from human malignancies has been induced by interleukin-2, *in vitro*, for periods of up to 60 days. These lymphocytes have demonstrated human antitumor activity in patients with lung cancer when administered without the concurrent intravenous administration of interleukin-2. See R. L. Kradin et al., Can. Immunol. Immunother., 24, 76 (1987).

Additional cytokines synthesized by T-cells include migration inhibition factor (inhibits the random migration of macrophages); leukocyte inhibition factor (inhibits the random migration of neutrophils); macrophage activation factor (enhances the cytolytic activity of macrophages); fibroblast activation factor (stimulates proliferation of fibroblasts); and interleukin-3 (IL-3) (activity similar to colony-stimulating factor).

Although the mechanistic details for cytokine activity are not known with certainty, the general mechanism for activity is believed to include the steps of: 1) binding of the cytokine to a specific cell surface receptor; 2) initiation of certain "cell surface activated" events; and 3) internalization of the cytokine-receptor complex where internal interactions result in proliferation, growth, differentiation, and/or the expression of specialized cell function.

Specifically, in the case of IL-2, the

interaction of IL-2 with T-cells is believed to involve an initial interaction with a low affinity receptor, IL2Rb, resulting in induction of a second receptor molecule, IL2Ra, that forms a high affinity complex with IL-2. Association of IL-2 with the high affinity complex results in proliferation. In this process of activation and proliferation there is an internalization of the IL-2-receptor complex and a subsequent decrease in the number of surface IL-2 receptors. See K. A. Smith, Science, 240, 1169 (1988).

It has been suggested that cell surface associated events are sufficient for activity and that internalization of the receptor-ligand complex is not required, at least in certain cases. Porcine insulin and murine alpha/beta interferon bound to Sepharose via cyanogen bromide activation have been reported to have biological activity, through cell surface associated events. See P. Cuatrecases, Proc. Nat. Acad. Sci. USA, 63, 450 (1969); H. Ankel et al., Proc. Nat. Acad. Sci. USA, 70, 2360 (1973); and C. Chaney et al., Proc. Soc. Exp. Biol. Med., 147, 293 (1974).

The accuracy of these reports has been doubted by persons in the art, particularly because of the known instability of the particular covalent bond formed. See W. H. Scouten, Methods in Enzymology, Klaus Mosbach, ed., Academic Press Pub., 135, 31 (1987). The question of the necessity of internalization has remained a debated issue. See E. DeMaeyer and J. DeMaeyer-Guignand, Interferon and Other Regulatory Cytokines, John Wiley and Sons Pub., 67-90 (1988).

The cost, availability, and toxicity of cytokines, such as IL-2, can be a limiting factor in the usefulness of the cytokine as a biologically active agent. Therefore, it would be desirable to be able to reuse and/or use less of a particular cytokine while retaining a substantial amount of their biological activity, with possibly decreased toxicity.

Therefore, a continuing need exists for modified cytokines that retain comparable, and in some cases, improved biological activity when compared with corresponding soluble or free cytokines, thus providing a biologically active cytokine that can be reused to stimulate biological activity and/or that can be used in significantly smaller quantities.

Brief Description of the Invention

The present invention provides immobilized cytokines comprising cytokines firmly bound to a solid, preferably biologically compatible, insoluble immobilizing support. The bound cytokine retains substantially the activity of the free cytokine when bound to the support. Accordingly, the bound cytokine is able to be utilized repeatedly (reused) to stimulate biological activity, and/or used in significantly smaller total quantities than the corresponding soluble or free cytokine.

Cytokines useful in the present invention include, but are not limited to, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, tumor necrosis factor (TNF), gamma-interferon, alpha-interferon, beta-interferon, erythropoietin (EPO), granulocyte colony stimulating factor (GCSF), murine granulocyte colony stimulating factor (MuGCSF), granulocyte-macrophage colony stimulating factor (GMCSF), murine granulocyte-macrophage colony stimulating factor (MuGMCSF), insulin-like growth factor I (ILGF-I), insulin-like growth factor II (ILGF-II), transformation growth factor beta (TGF-β), epidermoid growth factor (EGF), platelet derived growth factor (PDGF), and fibroblast growth factor-basic (FGFb). Preferred cytokines include those described in the Examples, and more preferrably IL-2, GMCSF, GCSF, EPO, TNF, FGFb, TGFb, EGF, and PDGF.

The cytokine is preferably bound to a biologically compatible, particulate support by means of

covalent bonding, preferably by means of a linking arm. The cytokine is preferably firmly bound to the support in a manner that allows the activity of the cytokine to be stabilized by the immobilization. The activity thus is durable and reusable.

As used herein, "substantially the activity of the free cytokine" means that at least one of the one or more active sites of a cytokine remains active, and will produce significant biological activity as a bound cytokine. In other words, because cytokines have multiple, often overlapping, biological or regulatory effects, a bound cytokine of the present invention may demonstrate one or more activities the same as, or similar to, that of the free cytokine. Thus, in demonstrating efficiency of an immobilized cytokine of the present invention, one or more biological activities may be stabilized through immobilization. Thus, at least one activity is preserved in the bound state, and in some cases may be enhanced by the binding of the cytokine to the support.

The structure or length of the linking arm may be varied to optimize the biological activity of the bound cytokine. Preferred linking arms comprise one or more linking groups selected from the group consisting of: (a) diamines, having the general formula NH_2-R^1-NH_2, where R^1 is a C_2-C_{20} alkyl group; (b) amino acids, having the general formula NH_2-R^2-CO_2H, where R^2 is a C_1-C_{20} alkyl group; and (c) dialdehydes, having the general formula OHC-R^3-CHO, where R^3 is a C_1-C_{20} alkyl group.

Useful supports include, but are not limited to, fibers, microspheres, beads, particles, membranes, sheets, and the like.

As used herein, "cytokine" refers to the natural or recombinant form of the cytokine, as well as to modified sequences, biologically active fragments or portions of cytokines, genetically or chemically

modified forms of a cytokine, biologically equivalent synthetic ligands, or mixtures thereof, which exhibit a substantially equivalent profile of bioactivity, or a portion of the original profile of bioactivity.

The present invention also provides methods of using the immobilized cytokines, both in vitro and in vivo, for the proliferation, growth, differentiation and/or expression of specialized cell function, including inducing growth of a cytokine-dependent cell line, such as an IL-2 dependent cell line, by contacting it with an effective amount of an immobilized cytokine of the invention.

Summary of the Drawings

Fig. 1 is a graphical depiction of the growth of CTLL-2 cells (DPM's x 10^{-3}) using IL-2 immobilized via a carboxyl group of the IL-2 as compared to IL-2 immobilized via an amino group of the IL-2.

Fig. 2 is a graphical depiction of the concentration dependence of immobilized IL-2 (μg IL-2 in initial coupling reaction) on the growth of CTLL-2 cells, a cytotoxic T-lymphocyte cell line, as determined by [^{3}H]-thymidine incorporation (DPM's x 10^{-3}).

Fig. 3 is a graphical depiction of the growth of CTLL-2 cells (DMP's x 10^{-3}) using immobilized IL-2 as a function of time (hours) relative to the growth of CTLL-2 cells using soluble IL-2.

Fig. 4 is a graphical depiction of the growth of human peripheral blood lymphocytes (PBL's in DPM's x 10^{-3}), using immobilized IL-2 as a function of time (hours) relative to the growth of PBL's using soluble IL-2.

Figs. 5A and 5B are graphical depictions of stimulation of granulopoiesis as measured by an increase in the white blood count of mice receiving soluble (5A) or immobilized (5B) MuGMCSF.

Fig. 6 is a graphical depiction of stimulation

of granulopoiesis as measured by the increase in white blood count of cyclophosphamide-treated mice receiving soluble or immobilized recombinant murine GMCSF (rMuGMCSF).

Fig. 7 is a graphical depiction of the stability of covalent bound rMuGMCSF as measured by its retention in contrast to adsorbed rMuGMCSF following SDS washes.

Detailed Description of the Invention

Cytokines

Interleukin-2 (IL-2) is commercially available as T-cell growth factor (human interleukin-2 recombinant; T3267) and as derived from cultured rat splenocytes (TD892) from Sigma Chemical Co., St. Louis, MO. Recombinant IL-2 (ala-125 analog and natural sequence) is also commercially available from Amgen, Thousand Oaks, CA. Natural sequence recombinant interleukin-3 (IL-3), natural sequence recombinant interleukin-4 (IL-4), and natural sequence recombinant interleukin-6 (IL-6) are commercially available from Amgen, Thousand Oaks, CA.

Recombinant human granulocyte-macrophage colony stimulating factor (rHuGMCSF), recombinant human granulocyte colony stimulating factor (rHuGCSF), recombinant human erythropoietin (rHuEPO), recombinant murine granulocyte- macrophage colony stimulating factor (rMuGMCSF), recombinant human gamma interferon (rHuIFN-gamma), and recombinant human epidermoid growth factor (rHuEGF) and fibroblast growth factor-basic (FGFb) all are available from Amgen, Thousand Oaks, CA. Recombinant human platelet derived growth factor (rHuPDGF), recombinant human insulin-like growth factor I (rHuILGF-I), recombinant human insulin-like growth factor II (rHuILGF-II), and transformation growth factor alpha (TGF-alpha) are commercially available from Bachem, Torrance, CA. Transformation growth factor

beta, porcine, (pTGF-beta) is commercially available from R & D Systems, Minneapolis, MN. Transformation growth factor beta is also commercially available from Collaborative Research, Bedford, MA. Recombinant interferon alpha is commercially available as Roferon™ from Roche Laboratories.

The biologically active portions of certain cytokines have also been isolated. The present invention also includes binding the biologically active portions of cytokines to a suitable support.

IL-1 has multiple effects on lymphocyte populations, including its function as an autocrine growth factor for many T-cell clones. IL-1 is also a potent stimulator of thymocyte proliferation, and of mitogen, nominal antigen plus Ia antigen, or alloantigen stimulated helper T-cells. IL-1 increases interleukin-2 receptor expression and IL-2 secretion of human peripheral T-cells in the presence of monoclonal antibody to the antigen-receptor complex. In addition, IL-1 acts as a cofactor for Con A activation of resting T-cells and is required for the proliferation of lymphocytes which express a high affinity receptor for IL-1. IL-1 is produced by human lung endothelium where it is believed to function in the development of inflammatory infiltrates.

IL-1 is also a regulator of hematopoietic activity. IL-1 induces endothelial cells to release granulocyte-macrophage colony stimulating factor (GMCSF) and granulocyte colony stimulating factor (GCSF), thus providing the mechanism by which IL-1 modulates granulocyte production and function during inflammation. IL-1 also releases GMCSF from monocytes and enhances growth factor dependent proliferation of human hematopoietic precursors.

IL-1 has demonstrated by its antitumor activity causing complete regression of relatively large immunogenic murine sarcomas by augmenting an ongoing

T-cell response. IL-1 has a direct cytotoxic effect in vitro on human A375 melanoma cells. IL-1 has also shown synergistic effects with interleukin-2 (IL-2) in the production of lymphokine activated killer cells.

This broad range of activity of IL-1 as a regulator of lymphocyte function, hematopoiesis, and lymphocyte antitumor activity has made IL-1 a valuable cytokine in a wide variety of in vivo and in vitro uses. See, for example, T. Hoang et al., J. Exp. Med., 168, 463 (1988); R. J. North et al., J. Exp. Med., 168, 2031 (1988); B. Tartakovsky et al., J. Immunol., 141, 3863 (1988); A. H. Lichtman et al., Proc. Nat. Acad. Sci. USA, 85, 9699 (1988); B. S. Bochner et al., J. Immunol., 139, 2297 (1987); and V. C. Broudy et al., J.Immunol., 139, 464 (1987).

Interleukin-3 (IL-3), also known as multicolony stem cell activating factor, or as multicolony stimulating factor, is a glycoprotein hematopoietic growth factor. IL-3 has a broad range of activity, due to its ability to stimulate both early stem cells, common to many myeloid cell lineages, as well as committed cells. IL-3 binds to a 140 kilodalton cell surface phosphoprotein. In primates, continuous infusion of IL-3 results in a delayed, modest increase in the white cell count. However, IL-3 has a marked synergistic effect on the response to subsequent treatment with low doses of granulocyte-macrophage colony stimulating factor, suggesting that IL-3 acts on early lineage cells that require a subsequent second factor to complete development. This hypothesis is consistent with tissue culture studies indicating that IL-3 is more effective in supporting colony formation by blast cells. In addition, IL-3 itself will not support in vitro colony formation, but requires a later acting factor, such as GMCSF. IL-3 acts synergistically with IL-6 to support early blast colony formation, with granulocyte colony stimulating factor (GCSF) to enhance

neutrophil formation, and with GMCSF to enhance granulocyte and macrophage colony formation. This broad range of activity as a hematopoietic cytokine makes IL-3 a valuable adjunct to hematopoietic cytokine therapy.

Like other cytokines, IL-3 also has negative regulatory effects, as evidenced by its inhibition of lymphokine activated killer cells. To date, IL-3 has been the only cytokine implicated in the regulation of early B-cell development, as is evidenced by the IL-3 dependent clones from murine fetal liver or adult bone marrow which show characteristics of B-cell precursors. See, for example, R. E. Donahue et al., Science, 241, 1820 (1988); R. J. Isfort et al., Proc. Nat. Acad. Sci. USA, 85, 7982 (1988); D. Rennick et al., J. Immunol., 142, 161 (1989); and G. Gallagher et al., Clin. Exp. Immunol., 74, 166 (1988).

Interleukin-4 (IL-4) is also known as B-cell stimulatory factor-1 (BSF-1), B-cell differentiation factor (BCDF), and B-cell growth factor 1 (BCGF-1). In the murine system, IL-4 enhances immunoglobulin IgG1 and IgE production in lipopolysaccharide activated cells, increases the expression of histocompatibility antigens on B-cells, and is required for the proliferation of anti-IgM activated B-cells.

In human studies a similar effect to that observed in the murine system on lymphocyte function has been described. High affinity receptors exist for IL-4 on both human hematopoietic and nonhematopoietic cells. IL-4 can induce proliferation in unstimulated thymocytes, and the response is strongly augmented with mitogens. IL-4 also augments the mitogen induced stimulation of human peripheral T-cells in the presence of dexamethasone, which inhibits IL-2 production. IL-4 also down modulates IL-2 induced human B-cell proliferation, and inhibits IL-2 induced NK cell activation and proliferation. IL-4, in conjunction with IL-2, however, augments the growth of tumor infiltrating

lymphocytes to autologous human malignant melanoma. In addition to its effects on lymphoid cells, IL-4 interacts with GMCSF and EPO to enhance granulocyte-macrophage and erythroid cell forming units. See, for example, H. Spits et al., J. Immunol., 139, 1142 (1987); Y. Kawakami et al., J. Exp. Med., 168, 2183 (1988); A. Nagler et al., J. Immunol., 141, 2349 (1988); A. Vazquez et al., J. Immunol., 142, 94 (1989); T. DeFrance et al., J. Exp. Med., 168, 1321 (1988); S. Karray et al., J. Exp. Med., 168, 85 (1988); and B. Brooks and R. C. Rees, Clin. Exp. Immunol., 74, 162 (1988).

Interleukin-6 (IL-6) is also known as B-cell stimulatory factor-2, interferon beta-2, and hybridoma-plasmacytoma growth factor. IL-6 is a multi-functional cytokine, initially described as a T-cell lymphokine with antiviral activity. IL-6 has been demonstrated to be produced by a variety of cells including: monocytes, fibroblasts, hepatocytes, cardiac myxomas, brain glial cells, and vascular endothelium. IL-6 activity is believed to include: regulation of fibroblast activity; acute phase protein production by hepatocytes; stimulation of human thymocytes and T-lymphocytes in the presence of mitogen; proliferation and differentiation of murine T-lymphocytes into cytotoxic cells; maintenance of myeloma derived cell lines; autocrine signalling for human multiple myeloma, and inhibition of the growth of carcinoma and leukemia/lymphoma cell lines. See, for example, P. B. Sehgal et al., Science, 235, 731 (1987); S. Shimizu et al., J. Exp. Med., 169, 339 (1989); J. L. Ceuppens et al., J. Immunol., 141, 3868 (1988); G. Tosato and S. E. Pike, J. Immunol., 141, 1556 (1988); M. Lotz et al., J. Exp. Med., 167, 1253 (1988); and L. Chen et al., Proc. Nat. Acad, Sci. USA, 85, 8037 (1988).

Granulocyte-macrophage colony stimulating

factor (GMCSF), granulocyte colony stimulating factor (GCSF), macrophage colony stimulating factor (MCSF) and multi-colony stimulating factor (IL-3) constitute a family of glycoproteins that have been recognized by their ability to stimulate and regulate the process of proliferation and differentiation of hematopoietic cells both _in vivo_ and _in vitro_. These individual cytokines are produced by one or more of the following cell sources: T-lymphocytes, monocytes, fibroblasts, epithelial cells, or endothelial cells. Additionally, IL-1, also known as hemopoietin-1, participates in this regulatory network by enhancing the effects of IL-3, MCSF, GCSF, and GMCSF.

Studies in animals have shown that GMCSF, GCSF, and IL-3 increase the number of functional white blood cells, and that the effect is enhanced by IL-1. Sequential administration of IL-3 and GMCSF has resulted in an increased platelet count as well. Use of members of this group of cytokines in nonhuman primates has shown benefit in viral induced pancytopenia, chemotherapy, and irradiation therapy induced myelo-suppression, leukopenia following whole body irradiation or high dose cytotoxic chemotherapy, and autologous bone marrow transplant.

In man the administration of both GCSF and GMCSF results in a significant increase in neutrophils and neutrophils-eosinophils respectively, as well as an increase in bone marrow cellularity with immature cells appearing in the blood. Clinical side effects following the use of GMCSF in man have included fever, rash, myalgia, fatigue, gastrointestinal distress, thrombophlebitis, bone pain, pleuritis, pleural effusion, pericarditis, and pulmonary emboli. The only side effect noted with GCSF has been bone pain.

The demonstrated benefits of GCSF and GMCSF in man have included: restoration of hematopoiesis following myelo-suppressive cytotoxic chemotherapy;

accelerated granulocyte recovery and decreased incidence of infection in patients with autologous bone marrow transplants; and improved circulating white cell, hemoglobin, and platelet count in patients with myelodysplastic syndrome and aplastic anemia. Administration of GMCSF to patients with AIDS associated leukopenia resulted in a significant increase in granulocytes and monocytes without increased viral production.

In addition to the hematopoietic effects described above, GMCSF has been demonstrated in vivo to activate monocytes to a tumorcidal state suggesting another potential clinical application for this cytokine. GMCSF has also been demonstrated to stimulate the proliferation in vitro of osteogenic sarcoma cell lines, a breast cancer cell line, a simian virus SV-40 transformed bone marrow stromal cell line and normal bone marrow fibroblast precursors. See, for example: S. Vadhan-Raj et al., N. Engl. J. Med., 319, 1628 (1988); J. E. Groopman et al., N. Engl. J. Med., 317, 593 (1987); K. H. Grabstein et al., Science, 232, 506 (1986); S. Dedhar et al., Proc. Nat. Acad. Sci. USA, 85, 9253 (1988); and A. A. Jakubowski et al., N. Engl. J. Med., 320, 38 (1989).

Erythropoietin (EPO) is the single cytokine required for the continued differentiation of the hematopoietic cells that produce mature red blood cells. In in vitro studies, the combination of IL-3, GMCSF or GCSF with EPO has been required for red cell production, suggesting that these cytokines are involved in the maintenance of the red cell precursor whereas EPO is required for the terminal differentiation and maturation. See, for example, J. Suda et al., Blood, 67, 1002 (1986).

Tumor Necrosis Factor (TNF), also known as a multi-functional cytokine produced by monocytes-macrophages, is a particularly important

mediator of inflammatory response. Two forms, TNF-alpha (Cachectin) and TNF-beta, exist. Among its effects TNF is a major factor in gram-negative endotoxin shock and induces a profound wasting (cachexia) syndrome in patients with cancer and chronic disease.

The range of activity includes stimulation of fibroblast growth, stimulation of osteoblast activity and bone reabsorption, promotion of angiogenesis, stimulation of collagenase and prostaglandin E_2 in synovial cells, and stimulation of procoagulant and platelet-activating factor in endothelial tissue.

TNF is an autocrine produced by macrophages. It functions as an immunomodulator, activating macrophages and increasing their ability to specifically recognize and kill malignant cells. TNF is chemotactic for macrophages, indicating its production at a site of inflammation both recruits and activates macrophages.

TNF participates in the cytokine network and induces the release of IL-1, GMCSF, platelet derived growth factor, and beta-2 interferon.

The major potential therapeutic effect of TNF is its antitumor activity. TNF is the mediator of endotoxin induced tumor regression. TNF may be involved in the antitumor activity of IL-2, since IL-2 induces TNF in human peripheral blood monocytes. TNF, given systemically, induces regression of tumor in mice. The direct anti-proliferative and tumor cytotoxic effects of TNF and IL-1 are believed synergistic.

Initial clinical studies of TNF in man have included intravenous and intramuscular injections. Toxicities have included: fever, chills, fatigue, anorexia, hypotension, and tachycardia. Several minor tumor responses have been noted to date. See, for example, B. Sherry and A. Cerami, J. Cell Biol., 107, 1269 (1988); J. J. Mule et al., Cancer Immunol. Immunother., 26, 202 (1988); Y. Ichinose et al., Cancer Immunol. Immunother., 27, 7 (1988);

P. B. Chapman et al., J. Clin. Onc., 5, 1942 (1987); H. H Bartsch et al., Mol. Biother., 1, 21 (1988); and T. Steinmetz et al., J. Biol. Resp. Mod., 7, 417 (1988).

Interferon (IFN) is a term originally assigned to a class of compounds discovered in the late 1950's with anti-viral activity. Originally, three classes of interferon were designated as alpha, beta, and gamma; designating their original identification and isolation from leukocytes, fibroblasts, and lymphoid cells, respectively. As of 1988, at least 24 nonallelic genes, coding for structurally related forms of alpha-interferon, had been described. These were divided into two subfamilies designated IFN-alpha I genes, which code for proteins of 165-166 amino acids, and IFN-alpha II genes, which code for protein of 172 amino acids. A single gene coding for what is commonly called "fibroblast interferon" has been fully characterized in humans. However, fibroblast can produce more than one form of interferon and the more correct term for fibroblast interferon is human interferon beta (HuIFN-beta). HuIFN-beta has about 40% amino acid homology with HuIFN-alpha. The human interferon gamma gene exists as a single copy with some individual to individual allelism or difference in single amino acids. Gamma-interferon has no homology to alpha- or beta-interferon.

Interferon, or virus-induced proteins with anti-viral activity, have been identified from representatives of all vertebral classes except amphibia. The biological activity of various IFN-alpha subtypes are relatively similar. The biological activity of INF-alpha and beta are also similar, but both differ from IFN-gamma. See E. DeMaeyer and J. DeMaeyer-Guignard, Interferon and Other Regulatory Cytokines, John Wiley and Sons, Pub., pp. 5-38 (1988). The major biological activities of alpha and beta interferon are: antiviral effects; induction of

monocytes to express major histocompatibility, complex class II antigens, and interleukin-1; antiproliferative effects; and regulation of human natural killer cell activity.

Interferon alpha and beta have an antitumor effect that involves a number of mechanisms including, among others, an antiproliferative effect, induction of differentiation, regulation of oncogene expression, and stimulation of the immune response.

The exact biological effects may vary with the particular structural forms of alpha-interferon and with the sensitivity of the assay cell line. It is also possible to observe both positive and negative regulation, as for example, human interferon alpha or beta may inhibit the maturation of monocytes to macrophages. See E. DeMaeyer and J. DeMaeyer-Guidnand in Interferon and Other Regulatory Cytokines, John Wiley and Sons, Pub., pp. 134-153 (1988).

A group of cytokines also termed growth factors have, among their biological activities, a positive or negative regulatory effect on wound healing and tissue repair including chemotactic activity, proliferation, growth and differentiation of epithelial cells and fibroblasts, stimulation of matrix formation and cartilage formation, and vascular formation (angiogenesis). A large number of biologically active proteins have been described within this area and have been classified on taxonomical principles into families and species based on their biological effects and amino acid sequence homology (as shown below in Table 1). Although this group of cytokines has been associated with tissue repair, they have other biological effects. In addition, other cytokines such as interleukin-1 and interleukin-3, which regulate immune responses, also have an effect on tissue repair.

Epidermal growth factor (EGF) is a key representative member of a family of structurally

related proteins including transformation growth factor (TGF) alpha, amphiregulin, and vaccinia growth factors. Human EGF was first isolated from urine and named urogastrone by its ability to inhibit gastric secretion (H. Gregory, Nature, 257, 324 (1975)). Murine EGF, isolated from the salivary gland is mitogenic for a large number of cell types including epithelial, fibroblasts, and endothelial cells (S. Nakagawa et al., Differentiation, 29, 284 (1985)). It stimulates precocious eyelid opening and tooth eruption in newborn mice (S. Cohen, J. B. Chem., 237, 1555 (1962)), and is chemotactic for epithelial cells (J. Blay and K. D. Brown, J. Cell Physiol., 124, 107 (1985)). EGF is synthesized as a precursor protein which is processed into a 53-amino acid active protein.

Transformation growth factor alpha (TGF-alpha) binds to the same receptor as EGF and shares similar biological activity. See G. J. Todaro et al., Proc. Nat. Acad. Sci. USA, 77, 5258 (1980). TGF-alpha, like EGF is synthesized as a 160-amino acid precursor, which is proteolytically processed into a 50-amino acid biologically active residue. See R. Derynck et.al., Cell, 38, 287 (1984). TGF-alpha was originally recognized by its ability to synergize with TGF-beta to induce anchorage independent growth of normal rat kidney fibroblast. See M. A. Azano et al., Proc. Nat. Acad. Sci. USA, 80, 6264 (1983).

Platelet derived growth factor (PDGF) is purified from human blood platelets. See R. Ross and A. Vogel, Cell, 14, 203 (1978). It consists of two polypeptide chains: the A chain (124 amino acid residues); and the B chain (140 amino acid residues). PDGF is a potent mitogen for cells of mesenchymal origin (e.g., smooth muscle and fibroblasts) but has no effect on epithelial or endothelial cells which lack PDGF receptors. See R. Ross, E. W. Raines, and D. F. Bowen-Pope, Cell, 45, 155 (1986). Platelet

derived growth factor may also be obtained from porcine cells.

Transformation growth factor beta(s) were originally identified by their ability to act synergistically with EGF or TGF-alpha to induce anchorage independent growth of NRK cells. See M. A. Anzano et al., Proc. Nat. Acad. Sci. USA, 80, 6264 (1983). They have subsequently been shown to have multiple biological effects including, chemotaxis, mitogenesis, growth inhibition and the induction or inhibition of differentiation depending upon other growth factors present. See M. B. Sporn and A. B. Roberts et al., J. Cell Biol., 105, 1039 (1987). In their mature form, TGF-betas are acid and heat-stable disulfide-linked homodimeric proteins of 112 amino acid residues which share 70% homology. See R. Derynck and J. A. Farrett et al., Nature, 316, 701 (1985). Another member of the family, beta-3, has recently been described. See J. M. Wozney and V. Rosen et al., Science, 242, 1582 (1988).

Although they share a variety of biological activities, different forms of TGF also possess unique biological activities for select target cells. See F. Rosa and A. B. Roberts et al., Science, 239, 783 (1988). TGF-beta I has demonstrated major activity in wound healing. Other biologically active proteins included in the TGF-beta family include: forms of gonadal proteins designated inhibin and activin that regulate pituitary secretion of follicle stimulating hormone; Mullerian inhibiting substances that cause regression of the female Mullerian ducts in the developing male embryo; and bone morphogenic proteins that are a group of polypeptides involved in the induction of cartilage and bone formation. See J. M. Wozney and V. Rosen et al., Science, 242, 1528 (1988).

Fibroblast growth factors (FGF) are

single-chain proteins of 14-18 kilodaltons. Two well characterized forms are basic FGF, isolated form brain and pituitary, and acidic FGF, isolated from brain and retina. Basic FGF, in most systems, is more stable and has ten times the potency of acidic FGF. Both forms of FGF bind to the same receptor and are mitogenic for cells of mesodermal origin such as fibroblasts, vascular endothelial cells, vascular smooth muscle, myoblasts, chondrocytes and osteoblasts. See F. Esch and A. Baird et al., Proc. Natl. Acad. Sci. USA, 85, 6507 (1985). The products of the int-2 and hst proto-oncogenes are also included as members of the FBF family. (See C. Dickson and P. E. Gordon, Nature, 326, 833 (1987).

Insulin-like growth factor I (ILG-I) also known as Somatomedia C, and Insulin-like growth factor II (ILG-II) represent a current nomenclature for a number of factors initially purified from serum and sharing the three biological activities of stimulating of sulfate incorporation into cartilage, insulin-like activity, and multiplication-stimulating activity. The liver and fibroblasts are major sources of circulating insulin-like growth factors, but essentially all tissues have been shown to produce them.

Insulin-like growth factors, among their biological activities, have also been shown to stimulate glucose metabolism, and stimulate DNA synthesis and cell proliferation of fibroblasts, sertoli cells, fetal brain cells, myoblasts, lens epithelium, pancreatic beta cells, lectin stimulating lymphocytes, and density arrested Balb/c 3T3 cells after being rendered "competent" with Platelet Derived Growth Factors. (See R. C. Baxter, Adv. Clin. Chem., 25, 50 (1986)).

Cytokines react with cell surface receptors which themselves are complex and may consist of subunits. Portions of the cytokine may bind preferentially to various subunits of the receptor resulting in different biological and/or regulatory

effects. The present invention also provides for immobilization of such cytokine fragments that may be directed to a particular subunit of the receptor.

Immobilizing Supports

Support materials useful in the present invention are preferably biologically compatible, and may be nonbiodegradable or biodegradable as desired. It may be desirable that the support be biodegradable when the bound cytokine will be utilized in vivo, while insoluble support materials are useful in applications such as bioreactors.

Suitable supports include fibers, sheets, microspheres, particles, beads, membranes, and the like.

The support preferably comprises a surface which is chemically compatible with the covalent attachment of the cytokine. Accordingly, the support preferably includes a surface having appropriate functional groups which can covalently bind to a site on the cytokine (e.g., an amino or carboxyl site), or to a suitable linking arm that can bind to a site on the cytokine. If the intended support does not have suitable functional groups for cytokine binding, such groups can be provided by appropriate chemical modification of the support surface. For example, a nonfunctionalized polystyrene support can be provided with a functionalized surface by suitable functionalization of the aromatic rings (e.g., via bromination).

Not all binding chemistries work equally well with each of the many various cytokines. Suitability of a particular binding chemistry used may, in part, depend upon the availability of reactive sites, and their proximity to the active site of the cytokine. Those skilled in the art can, however, reasonably predict a suitable approach from the amino acid sequence, the presence of reactive groups, and the active site. In

applying the invention, those skilled in the art can also create genetically modified cytokines replacing amino acids with non-reactive amino acids, or vice versa to target linkage of the immobilization site. Those skilled in the art may also modify the codon of the cytokine to produce one with terminal reactive groups thereby providing a high probability of directing linkage of the immobilization site.

A functionalized surface includes reactive functional groups that provide a site for binding: (a) directly to a site on the cytokine; or (b) to a suitable linking arm. Such functional groups include hydroxyl (-OH), amino ($-NH_2$ or -NHR, wherein R is alkyl or aryl), carboxyl ($-CO_2H$), sulfhydryl (-SH), and halogens (-F, -Cl, -Br, -I). A functionalized surface may be provided by a number of means in addition to chemical treatment of a surface. For example, blue-dyed polystyrene beads obtained from Polysciences, provide a functionalized surface despite polystyrene itself not having functional groups available for reaction. The blue dye is bound to, adsorbed on, or copolymerized with the polystyrene and provides free amino groups. A wide variety of other methods for providing suitable functional groups are known.

Suitable particulate supports include inorganic supports, such as, glass, quartz, ceramics, zeolites, metals, and metal oxides; polymeric materials, including homopolymers, copolymers, and oligopolymers, derived from monomeric units comprising definite units such as styrene, divinylbenzene, ethylene, butadiene, acrylonitrile, acrylic acid, methacrylic acid, esters of acrylic and methacrylic acid, vinyl acetate, fluoroalkene, acrylamide, and methacrylamide; carbohydrate supports, such as, agarose, cross-linked agarose, dextran, cross-linked dextran, inulin, hyaluronic acid, cellulose, cellulose derivatives such as carboxymethyl cellulose (CMC), starch and starch

derivatives (e.g., starch microspheres); and insoluble protein materials, such as, gelatin, collagen, or albumin.

The surface of the immobilizing support of the present invention is preferably nonporous. The use of materials having a nonporous surface, such as substantially spherical polymeric beads or microspheres, allows for binding of the cytokine to the outer surface of the support, thereby providing the cytokine in a biologically available, unhindered position. A surface is considered nonporous where the size of any pores in the material is sufficiently small so as to block or substantially hinder the migration of the cytokine into the interior of the spheres. For use as a sustained release, biodegradable formulation, a porous surface may be preferred to permit high drug loading, with new active sites exposed as the support degrades.

The size and shapes of the support may be varied widely, depending on the particular cytokine and its intended use. Polymeric spheres having a diameter of about 0.5-500 μm, and particularly about 1-75 μm, are preferable supports. Such supports are preferred, for example, for the <u>in vitro</u> growth of IL-2 dependent lymphocytes. Other preferable supports include staple fibers having a diameter of about 5-200 μm.

<u>Cytokine Linking Groups</u>

The immobilized cytokines of the present invention preferably include a cytokine covalently bound, either directly or through a linking arm, to the support materials. It is believed that the length of the linking arm may be related to the biological activity of the bound cytokine. Suitable linking arms include one or more bifunctional linking groups such as: (1) diamines, having the formula NH_2-R^1-NH_2, where R^1 is a C_2-C_{20} alkyl group; (2) amino acids, having the general formula NH_2-R^2-CO_2H, where R^2 is a C_1-C_{20} alkyl

group; and (3) dialdehydes, having the formula OHC-R^3-CHO, where R^3 is a C_1-C_{20} alkyl group. Two or more linking groups may be coupled to provide additional length. Examples of suitable linking groups include 6-aminocaproic acid, 1,6-diaminohexane, 1,12-diaminododecane, glutaraldehyde, and mixtures thereof.

In a preferred embodiment of the invention, the solid support includes a functionalized surface having a plurality of reactive, exposed functional groups. The cytokine is thus directly covalently attached to a functional group on the surface, or to a linking arm of appropriate length that is covalently attached to the functional group. Following extensive washing of the support having a functionalized surface, the biologically active moiety (cytokine) is attached to an exposed functional group or to the linking arm. Acceptable methods of attachment include: (1) the use of water-soluble carbodiimides in the reaction of a carboxyl group on the functionalized surface of the polymeric support and a free accessible amino group on the cytokine, believed to form a stable amide bond; (2) the use of bifunctional aldehydes (e.g., glutaraldehyde) as a linking arm, which can couple an amino group on the surface of the polymeric support and a free accessible amino group on the cytokine; and (3) the use of cyanogen bromide in the reaction of a hydroxyl group on the solid support with an amino group on a linking arm or on the cytokine.

The stability of the immobilized cytokine will depend on the nature of the covalent bond(s) between the cytokine, either directly to the immobilizing surface, or through the linking arm (if present). Stable, firmly bound cytokines will demonstrate the desired biological activity through repeated uses.

The stability of the following bonds linking a protein to an insoluble matrix are considered relatively weak:

1. <u>Isourea</u> ($\text{Polymer-O-}\overset{\overset{\text{NH}}{\|}}{\text{C}}\text{-NH-Protein}$)

This linkage is formed from the reaction of an amino group on the protein (chiefly the lysyl side chain amine) with polyhydroxylic matrices (e.g., agarose, cellulose, and dextran) that have been activated with such reagents as cyanogen bromide (CNBr), 1-cyano-4-N,N-dimethylamine pyridinium tetrafluoroborate, and the like.

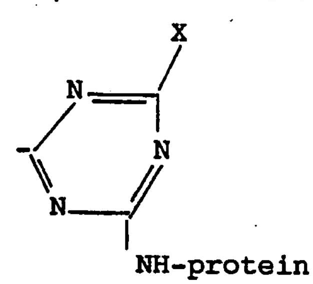
2. <u>Imidocarbonate</u> ($\text{Polymer-O-}\overset{\overset{\text{N-Protein}}{\|}}{\text{C}}\text{-O-Polymer}$)

This linkage is also formed as above for isourea, from the reaction of an amino group on the protein (chiefly the lysyl side chain) with polyhydroxylic matrices activated as above.

The stabilities of the following protein-insoluble matrix bonds are considered relatively strong:

1. <u>Urethane</u> ($\text{Polymer-O-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-NH-Protein}$)

This linkage is formed from the reaction of an amino group on the protein with polyhydroxylic matrices that have been activated with such reagents as 4-nitrophenyl chloroformate, N-hydroxysuccinimidyl chloroformate, carbonyl diimidazole, and the like.

2. <u>Triazine ether</u> (Polymer-O-C 

This linkage is formed from the reaction of a protein amino group with a polyhydroxylic matrices that have been activated with such reagents as cyanuric chloride.

The stability of the following protein-insoluble matrix bonds are considered relatively very strong:

1. <u>Amine</u> (Polymer-NR-Protein)

This linkage is formed in a variety of ways, including the reaction of a protein amino group with (1) polyhydroxylic matrices that have been activated with such reagents as tresyl chloride, sulfonyl chloride and the like, with oxiranes (epoxides) such as bisoxirane and epichlorohydrin and with (2) polyamino matrices that have been activated with such reagents as glutaraldehyde.

2. <u>Amide</u> (Polymer-C(=O)-NH-Protein or Polymer-NH-C(=O)-Protein)

This linkage can be formed in a variety of ways, including the reaction of a protein amino group with an activated carboxyl group on an insoluble matrix. Activation of these carboxyl groups can be achieved via formation of "active" esters (e.g., N-hydroxysuccimimidyl, *p*-nitrophenol, or pentachlorophenol) or by reaction with carbodiimides. Conversely, an amide bond may also be formed by the reaction of an amino group on an insoluble matrix with a suitably activated (e.g., a water soluble carbodiimide) carboxyl group on the protein, especially the aspartic acid and glutamic acid side chain carboxyl groups.

It is preferred that the covalent attachment be directed to a single site on the cytokine, preferably a suitable distance from the biologically active site. This consideration may dictate the preferred choice of linking arms and the specific chemistry chosen in the attachment

of the linking arms, support, and cytokine to optimize biological activity.

Use of the Bound Cytokine

Bound cytokines of the present invention can be used to induce and regulate a variety of biological reactions, including for example: (1) in vitro growth and production of cellular blood components including stem cells, and cells in various stages of differentiation, including red cells, lymphocytes, macrophages, and/or neutrophils; (2) the in vitro growth and production of specialized effector cells, including lymphokine activated killer (LAK) cells, natural killer cells, subpopulations of lymphokine activated killer cells, tumor infiltrating lymphocytes, and/or cytotoxic T-cells; (3) the treatment of malignant disease by the in vivo intraperitoneal and/or intrapleural administration of the bound cytokines; (4) the treatment of malignant disease by the in vivo intravenous administration of the bound cytokines; (5) the treatment, preferably by intravenous administration or by in-situ placement of the bound cytokine, of refractory anemias, thrombocytopenias, and neutropenias associated with primary bone marrow failure or secondary bone marrow failure due, for example, to a lack of erythropoietin in chronic renal failure and/or renal failure in patients on renal dialysis; (6) the treatment of hard and soft tissue wounds by surface application of the bound cytokines or in-situ placement of the bound cytokines; and (7) treatment of osteoporosis by the in vivo intravenous administration of the bound cytokines. See S. Nakagawa and S. Yoshida et al., Differentiation, 29, 284 (1985), and J. Blay and K.D. Brown, J. Cell Physiol., 124, 107 (1985).

The present invention will be further described by reference to the following examples.

Example 1

Attachment of IL-2 to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant IL-2 (Amgen, Thousand Oaks, CA, ala-125 analog) was immobilized on 9.64 μm blue-dyed polystyrene beads (Polysciences, Warrington, PA) using a bifunctional aldehyde in the following manner. A 0.25 ml-aliquot of a 2.5% aqueous suspension of 9.64 μm blue-dyed polystyrene beads was diluted with 1.0 ml of phosphate buffered saline (PBS, pH 7.40) and centrifuged for 5 minutes in a microcentrifuge. The supernatant was carefully removed and discarded. The beads were washed twice by suspension in 1.0 ml-portions of PBS followed by centrifugation. The beads were then suspended in 0.75 ml of an 8% solution of glutaraldehyde in PBS. Activation was allowed to proceed for 5 hours at room temperature with gentle end-over-end mixing. The reaction mixture was centrifuged and the supernatant was discarded. The pellet, i.e., the agglomerated beads, was washed twice with 1.0 ml-portions of PBS. The pellet was then suspended in 0.4 ml of PBS and treated with 0.1 ml of an aqueous IL-2 solution (100 μg IL-2, activity 600,000 units). The reaction mixture was mixed overnight at room temperature, centrifuged, and the supernatant was carefully removed and saved. The pellet was resuspended in 0.5 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. This combined supernatant solution (ca. 1.0 ml) was preserved at 4°C for the subsequent determination of residual IL-2 activity.

The beads were then processed in the following manner. The beads were suspended in 0.5 ml of 0.5 M ethanolamine in PBS and mixed for 30 minutes at room temperature. The mixture was centrifuged, the supernatant was discarded, and the pellet was washed once with 0.5 ml of PBS. The beads were suspended in 0.5 ml of 1% bovine serum albumin (BSA, Sigma, St. Louis, Mo) in PBS, mixed for 30 minutes at room temperature, and centrifuged. The

supernatant was discarded. The pellet was then washed twice with 0.5 ml-portions of the BSA/PBS solution and finally suspended in 0.5 ml of a storage buffer (sodium chloride (0.88%), BSA (1%), glycerol (5%), and sodium azide (0.1%) in 0.02 M sodium phosphate (pH 7.40)). The beads were stored at 4°C until used.

An assay of the supernatant solution for IL-2 activity revealed an activity of 50,400 units (8.4% of the activity of the original solution), indicating that 91.6% of the IL-2 had been bound to the beads.

Example 2

Attachment of IL-2 to Blue-Dyed Polystyrene Beads (0.93 μm)

Recombinant IL-2 (Amgen, ala-125 analog, 100 μg IL-2, activity 660,000 units) was immobilized on 0.93 μm blue-dyed polystyrene beads (Polysciences) using a bifunctional aldehyde following the procedure described in Example 1. Because of the smaller bead size, however, longer centrifugation times (10 minutes) were required to effect the complete separation of the beads from the supernatant. Following the final washes, the beads were suspended in 0.5 ml of the storage buffer used in Example 1 and kept at 4°C until used. An assay of the supernatant solution for IL-2 activity revealed an activity of 18,000 units (2.7% of the activity of the original solution), indicating that 97.3% of the IL-2 had been bound to the beads.

Example 3

Attachment of IL-2 to Blue-Dyed Polystyrene Particles (421 μm)

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on blue-dyed polystyrene particles (Polysciences, 421 μm) using a bifunctional aldehyde in the following manner. Blue-dyed polystyrene particles (10 mg) were washed three times with 1.0 ml-portions of PBS (pH 7.40). They were then activated with glutaraldehyde

and coupled to recombinant IL-2 (0.2 ml of aqueous IL-2 solution, 200 μg IL-2, activity 1.5 x 10^6 units) following the procedure described in Example 1. Following coupling and processing as described in Example 1, the beads were stored in 1.0 ml of the storage buffer used in Example 1 at 4°C. A determination of the IL-2 activity in the supernatant revealed an activity of 176,000 units (11.7% of the activity of the original solution), indicating that 88.3% of the IL-2 had been bound to the particles.

Example 4

Attachment of IL-2 to Blue-Dyed Polystyrene Beads (9.64 μm): Effect of IL-2 Solution Concentration

The effect of IL-2 (Amgen, ala-125 analog) concentration in the immobilization process was demonstrated in the following manner. The pellets obtained from eight 0.125 ml-aliquots of a 2.5% aqueous suspension of blue-dyed polystyrene beads (9.64 μm) were washed with PBS and activated with glutaraldehyde as described in Example 1, except that the reactions were carried out at one-half the scale. The activated beads were then suspended in various amounts of PBS and IL-2, as designated in Table 2, and allowed to react at room temperature overnight. Following this coupling reaction, the beads were processed according to the procedure described in Example 1, suspended in 0.25 ml-portions of the storage buffer, and kept at 4°C until used. The supernatant solutions obtained from the various coupling reactions were assayed for the residual IL-2 activity present. The results appear in Table 2. The difference between the activity of each IL-2 solution used in the coupling reactions (before) and that recovered in the resultant supernatant (after) yielded the value of % IL-2 incorporated.

Example 5

Attachment of IL-2 (Recombinant Natural Sequence) to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant IL-2 (Amgen, natural sequence) was immobilized on 9.64 μm blue-dyed polystyrene beads following a procedure similar to that described in Example 1. The pellet obtained from a 0.125 ml-aliquot of a 2.5% aqueous suspension of blue-dyed polystyrene beads was washed with three 0.5 ml-portions of PBS, activated with 0.5 ml of 8% glutaraldehyde/PBS, and suspended in a solution of recombinant IL-2 (0.032 ml of aqueous IL-2 solution, 32 μg IL-2, activity 60,000 units) in 0.4 ml of PBS. After allowing the reaction to proceed by mixing at room temperature overnight, the reaction mixture was centrifuged and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.5 ml of PBS and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were processed following the procedure described in Example 1, suspended in 0.25 ml of the storage buffer, and kept at 4°C until used. A determination of the IL-2 activity in the supernatant revealed an activity of 5,700 units (9.5% of the activity of the original solution), indicating that 90.5% of the IL-2 had been bound to the beads.

Example 6

Attachment of IL-2 to Polybead® Carboxylate Microspheres (9.67 μm)

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 9.67 μm Polybead® carboxylate microspheres (Polysciences, carboxylate modified polystyrene) using a water-soluble carbodiimide in the following manner. The pellet obtained from a 0.25 ml-aliquot of a 2.5% aqueous suspension of Polybead® carboxylate microspheres was washed with three 1.0 ml-portions of PBS. The beads were

suspended in 0.4 ml of PBS, and 3.0 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDCI, Pierce Chemicals, Rockford, IL) was added and dissolved. An aqueous solution of recombinant IL-2 (0.05 ml, 50 µg IL-2, activity 375,000 units) was then added. After mixing overnight at room temperature, the reaction mixture was centrifuged and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.5 ml of PBS and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then processed according to the method described in Example 1, suspended in 0.25 ml of the storage buffer, and stored at 4°C until used. An assay for IL-2 activity in the supernatant revealed an activity of 570 units (0.2% of the activity of the original solution), indicating that 99.8% of the IL-2 had been bound to the beads.

Example 7

Attachment of IL-2 to Polybead® Carboxylate Microspheres (9.67 µm) With a 6-Aminocaproic Acid Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 9.67 µm Polybead® carboxylate microspheres with a 6-aminocaproic acid linking arm using a water-soluble carbodiimide in the following manner. The pellet obtained from a 0.25 ml-aliquot of carboxylate microspheres was washed as described in Example 6, suspended in 0.5 ml of PBS, and treated with 3.0 mg of N-hydroxysulfosuccinimide (sulfo-NHS, Pierce Chemicals, Rockford, IL) and 3.0 mg of EDCI. After vortexing to dissolve the reagents, the reaction mixture was gently mixed for 30 minutes at room temperature. The slurry was then centrifuged and the supernatant was discarded. The pellet was suspended in 0.5 ml of a 0.5 M solution of 6-aminocaproic acid in PBS. The resulting slurry was mixed for 20 hours at room temperature and centrifuged. The supernatant was discarded. The pellet was washed with three 0.5 ml-portions of PBS, resuspended in 0.35 ml of

PBS, and treated with 0.05 ml of an aqueous solution of IL-2 (50 μg IL-2, activity 375,000 units) and 2.0 mg of EDCI. After vortexing to dissolve the reagents, the reaction mixture was gently mixed at room temperature overnight. The slurry was then centrifuged, and the supernatant was carefully removed and saved. The pellet was resuspended in 0.6 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed an activity of 460 units (0.1% of the original solution), indicating that 99.9% of the IL-2 had been bound to the beads.

Example 8
Attachment of IL-2 to Polybead® Carboxylate Microspheres (9.67 μm) With a 1,6-Diaminohexane/Glutaraldehyde Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 9.67 μm Polybead® carboxylate microspheres with a 1,6-diaminohexane/glutaraldehyde linking arm using a water-soluble carbodiimide in the following manner. The pellet obtained from a 0.25 ml-aliquot of carboxylate microspheres was washed with three 1.0 ml-portions of PBS (pH 7.40), suspended in 0.5 ml of a 0.5 M solution of 1,6-diaminohexane in PBS (pH 9.50) and treated with 3.0 mg of EDCI. The slurry was vortexed to dissolve the reagents and mixed for 20 hours at room temperature. This reaction mixture was centrifuged, the supernatant was discarded, and the pellet was washed with three 0.5 ml-portions of PBS (pH 7.40). The pellet was then suspended in 0.5 ml of 8% glutaraldehyde in PBS and mixed for 4 hours at room temperature. The slurry was centrifuged, the supernatant was discarded, and the pellet was washed again with three 0.5 ml-portions of PBS. The resulting pellet was then

suspended in 0.35 ml of PBS and treated with 0.05 ml of an aqueous solution of IL-2 (50 μg IL-2, activity 375,000 units). The slurry was mixed overnight at room temperature, centrifuged, and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.6 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed 50,000 units (13.3% of the original), indicating that 86.7% of the IL-2 had been bound to the beads.

Example 9

Attachment of IL-2 to Polybead® Carboxylate Microspheres (9.67 μm) With a 1,12-Diaminododecane/Glutaraldehyde Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 9.67 μm Polybead® carboxylate microspheres with a 1,12-diaminododecane/glutaraldehyde linking arm using a water-soluble carbodiimide in the following manner. The pellet obtained from 0.25 ml of carboxylate microspheres was washed with PBS (pH 7.40, 3 x 1.0 ml), suspended in 0.75 ml of 0.2 M 1,12-diaminododecane in PBS (pH 7.0), and treated with 5.0 mg of EDCI. After mixing for 18 hours at room temperature, the reaction mixture was centrifuged and the supernatant was discarded. The pellet was washed with PBS (pH 7.40, 3 x 1.0 ml), and activated with 1.0 ml of 8% glutaraldehyde in PBS as described in Example 8. After activation, the slurry was centrifuged, the supernatant was discarded, and the pellet was washed again with three 0.5 ml-portions of PBS. The resulting pellet was then suspended in 0.4 ml of PBS, treated with 0.1 ml of an aqueous IL-2 solution (100 μg IL-2, activity 750,000 units). The mixture was allowed to react overnight at room temperature. The slurry was centrifuged

and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.5 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant showed an activity of 42,000 units (5.6% of the original), indicating that 94.4% of the IL-2 had been bound to the beads.

Example 10

Attachment of IL-2 to Polybead® Carboxylate Microspheres (65 ± 25 μm) With a 1,12-Diaminododecane/Glutaraldehyde Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 65 ± 25 μm Polybead® carboxylate microspheres (Polysciences) with a 1,12-diaminododecane/glutaraldehyde linking arm using a water-soluble carbodiimide in the following manner. The pellet obtained from 0.50 ml of a 2.5% suspension of 65 ± 25 μm carboxylated polybeads was washed with PBS (pH 7.40, 3 x 1.0 ml), suspended in 1.0 ml of 0.2 M 1,12-diaminododecane in PBS (pH 6.00), and treated with 10 mg of EDCI. After mixing for 24 hours at room temperature, the reaction mixture was centrifuged and the supernatant was discarded. The pellet was washed with PBS (pH 7.40, 3 x 1.0 ml), and activated with 1.0 ml of 8% glutaraldehyde in PBS as described in Example 8. After activation, the slurry was centrifuged, the supernatant was discarded, and the pellet was washed again with three 0.5 ml-portions of PBS. The resulting pellet was then suspended in 0.75 ml of PBS, and treated with 0.25 ml of an aqueous IL-2 solution (0.1025 mg IL-2, activity 900,000 units). The mixture was allowed to react by mixing overnight at room temperature. The beads were processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until

used. A determination of the IL-2 activity present in the supernatant revealed an activity of 144,450 units (16.0% of the original), indicating that 84% of the IL-2 had been bound to the beads.

Example 11
Attachment of IL-2 to Polybead® Carboxylate Microspheres (9.67 μm) With a 1,12-Diaminododecane Linking Arm via Free Carboxyl Groups on the Cytokine

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 9.67 μm Polybead® carboxylate microspheres with a 1,12-diaminododecane linking arm via free carboxyl groups on IL-2 using a water-soluble carbodiimide in the following manner. The pellet obtained from 0.25 ml of carboxylate microspheres (9.67 μm) was washed with PBS (pH 7.40, 3 x 1.0 ml), and reacted with 1,12-diaminododecane/EDCI as described in Example 9. After mixing for 18 hours at room temperature, the reaction mixture was centrifuged and the supernatant was discarded. The modified beads were then thoroughly washed with PBS (pH 7.40, 3 x 1.0 ml), resuspended in 0.4 ml of PBS, treated with 0.1 ml of an aqueous IL-2 solution (41 μg IL-2, activity 360,000 units) followed by 5.0 mg of EDCI, and mixed overnight at room temperature. The reaction mixture was centrifuged and the supernatant was carefully removed and saved. The pellet was resuspended in 0.5 ml of PBS and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then suspended in 1.0 ml of 1% BSA/PBS and mixed for 30 minutes at room temperature. The mixture was centrifuged and the supernatant was discarded. The pellet was washed with the BSA/PBS solution (3 x 1.0 ml) and finally suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed 834 units (0.2% of the original), indicating that 99.8% of the IL-2

had been bound to the beads.

Example 12

Attachment of Polyethylene Glycol-Modified IL-2 to Polybead® Carboxylate Microspheres (9.67 μm) With a 1,12-Diaminododecane Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was reacted with a ten-fold molar excess of methoxypolyethylene glycolyl N-succinimidyl glutarate (MW 4800) [Abuchowski et al., Cancer Biochem. Biophys., 1, 175 (1984)] following the procedure described by Katre and Knauf in International Patent Application Number PCT/US86/01252 (International Publication Number WO87/00056), incorporated herein by reference. The modified IL-2 was purified by size exclusion chromatography on a Bio-Gel P-10 column using PBS (pH 7.40) as the eluting solvent. The purified column fraction used for this experiment contained 764,000 units of IL-2 activity per ml of buffer.

The modified IL-2 was immobilized on 9.67 μm Polybead® carboxylate microspheres using a 1,12-diaminododecane linking arm in the following manner. The pellet obtained from 0.15 ml of carboxylate microspheres was reacted with 1,12-diaminododecane in the presence of EDCI following the procedure described in Example 9. After mixing for 18 hours at room temperature, the reaction mixture was centrifuged and the supernatant was discarded. The modified beads then were thoroughly washed with PBS (pH 7.40, 3 x 1.0 ml), resuspended in 0.3 ml of PBS, treated with 0.3 ml of the modified IL-2 solution (activity 229,000 units) followed by 5.0 mg of EDCI, and allowed to mix at room temperature overnight. The slurry was centrifuged and the supernatant was carefully removed and saved. The pellet was resuspended in 0.5 ml of PBS and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then suspended in 1.0 ml of

1% BSA/PBS and mixed for 30 minutes at room temperature. The mixture was centrifuged and the supernatant was discarded. The pellet was washed with the BSA/PBS solution (3 x 1.0 ml) and finally suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed 509 units (0.2% of the original), indicating that 99.8% of the IL-2 had been bound to the beads.

Example 13

Attachment of IL-2 to Polybead® Amino Microspheres

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 5.29 μm Polybead® amino microspheres (Polysciences, amino functionalized polystyrene) using a bifunctional aldehyde in the following manner. The pellet obtained from a 0.25 ml-aliquot of Polybead amino microspheres was washed with PBS (3 x 0.5 ml), activated with 0.7 ml of 8% glutaraldehyde in PBS following the procedure described in Example 1. After washing the beads with PBS (3 x 0.5 ml), they were suspended in 0.4 ml of PBS, and treated with 0.1 ml of an aqueous IL-2 solution (100 μg IL-2, 750,000 units). The mixture was mixed overnight at room temperature. The reaction mixture was then centrifuged and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.5 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed 44,500 units (5.9% of the original solution), indicating that 94.1% of the IL-2 had been bound to the beads.

Example 14
Attachment of IL-2 to Sephadex® G-10 Particles (40-120 μm) With a 6-Aminocaproic Acid Spacer Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on degradable Sephadex® G-10 resin particles (Pharmacia, Piscataway, NJ, cross-linked dextran particles, 40-120 μm) with a 6-aminocaproic acid linking arm in the following manner. A slurry of approximately 7.5 ml of wet, packed Sephadex® G-10 resin in 7.5 ml of water was activated with 1.5 g of cyanogen bromide (CNBr) according to the published procedure; see P. Cuatrecasas, J. Biol. Chem., 245, 3059 (1970), incorporated herein by reference. After activation, the resin was rapidly filtered, washed with 100 ml of cold 0.2 M sodium borate buffer (pH 9.0), and added to 50 ml of 1.0 M 6-aminocaproic acid in 0.2 M sodium borate (pH 9.0). The mixture was mixed at room temperature for 20 hours. The resin was collected by filtration, washed with ca. 200 ml of H_2O, and dried under high vacuum for 48 hours. A 10 mg-portion of the dried resin was swollen for 24 hours in 1.0 ml of PBS. The suspension was then centrifuged, the supernatant discarded, and the resin washed with PBS (3 x 1.0 ml). The pellet was resuspended in 0.4 ml of PBS, treated with 0.1 ml of an aqueous solution of IL-2 (100 μg IL-2, activity 750,000 units), followed by 3.0 mg of EDCI, and mixed overnight at room temperature. The resin was processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant 852 units (0.1% of the original), indicating that 99.9% of the IL-2 had been bound to the resin.

Example 15

Attachment of IL-2 to Sephadex® G-10 With a 1,6-Diaminohexane/Glutaraldehyde Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on degradable Sephadex® G-10 particles with a 1,6-hexanediamine/glutaraldehyde linking arm in the following manner. Wet, packed Sephadex® G-10 resin (ca. 7.5 ml) was activated with CNBr following the procedure described in Example 14. The washed activated resin was then added to 50 ml of 1.0 M 1,6-hexanediamine in 0.2 M sodium borate (pH 9.0). The slurry was mixed at room temperature for 20 hours. The resin was collected by filtration, washed with 200 ml of H_20, and dried under high vacuum for 48 hours. A 10 mg portion of the dried resin was swollen and washed, as described in Example 12. The pellet was activated with 1.0 ml of 8% glutaraldehyde in PBS as described in Example 8. After activation, the slurry was centrifuged, the supernatant was discarded, and the pellet was washed again with three 0.5 ml-portions of PBS. The activated resin was suspended in 0.4 ml of PBS, and treated with 0.1 ml of an aqueous solution of IL-2 (100 μg IL-2, activity 750,000 units). The mixture was allowed to react with mixing overnight at room temperature. The slurry was then centrifuged and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.5 ml of PBS and the suspension was centrifuged. The supernatant was removed and added to the first supernatant. The resin was then processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity remaining in the supernatant revealed an activity of 29,800 units (4.0% of the original), indicating that 96.0% of the IL-2 had been bound to the resin.

Example 16
Attachment of IL-4 to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant IL-4 (Amgen, natural sequence) was immobilized on 9.64 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.25 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), and then activated with glutaraldehyde as described in Example 1. The beads were then suspended in 1.0 ml of a commercial IL-4 formulation containing 10.0 μg IL-4 (activity 2 x 10^5 units) and 0.025% human serum albumin (HSA) in PBS. The reaction mixture was mixed overnight at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used. A determination of the IL-4 activity present in the supernatant obtained from the above coupling reaction could not be measured due to the lack of a quantifiable assay.

Example 17
Attachment of IL-6 to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant IL-6 (Amgen, natural sequence) was immobilized on 9.64 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.25 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), and then activated with glutaraldehyde as described in Example 1. The beads were then suspended in 1.0 ml of a commercial IL-6 formulation containing 10.0 μg IL-6 (activity 1-2 x 10^5 units) and 0.025% HSA in PBS. The reaction mixture was mixed overnight at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of storage buffer and kept at 4°C until used. An assay of the supernatant solution from the above coupling reaction for IL-6 activity could not be quantified due to the lack of a suitable indicator cell

line.

Example 18
Attachment of Murine Granulocyte-Macrophage Colony Stimulating Factor to Blue-Dyed Polystyrene Beads (0.93 μm)

Recombinant murine granulocyte-macrophage colony stimulating factor (rMuGMCSF, Amgen) was immobilized on 0.93 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.25 ml of a 2.5% suspension of 0.93 μm blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), and then activated with glutaraldehyde following the procedure described in Example 2. The beads were then suspended in 0.5 ml of a commercial rMuGMCSF formulation containing 5.0 μg of the growth factor (activity 5 x 10^3 units) and 0.025% BSA in PBS. The reaction mixture was mixed overnight at room temperature. Following the final washes, the beads were suspended in 0.5 ml of the storage buffer and kept at 4°C until used. An assay of the supernatant solution for rMuGMCSF could not be quantified due to the unavailability of an indicator cell line.

Example 19
Attachment of Human Granulocyte-Macrophage Colony Stimulating Factor to Blue-Dyed Polystyrene Beads (0.93 μm)

Recombinant human granulocyte-macrophage colony stimulating factor (rHuGMCSF, Amgen) was immobilized on 0.93 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.125 ml of a 2.5% suspension of 0.93 μm blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), and then activated with glutaraldehyde as described in Example 2. The beads were then suspended in 0.6 ml of a commercial rHuGMCSF formulation that contained 3.0 μg of the growth factor (activity 120,000 units) and 0.025% HSA in PBS. The reaction mixture was mixed overnight at room temperature. Following the final washes, the beads were suspended in

0.5 ml of storage buffer and kept at 4°C until used. An assay of the supernatant solution for GMCSF activity revealed 46 units (0.04% of the original), indicating that 99.96% of human GMCSF had been bound to the beads.

Example 20
Attachment of IL-3 to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant IL-3 (Amgen, natural sequence) was immobilized on 9.64 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.25 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), and activated with glutaraldehyde following the procedure described in Example 1. The beads were then suspended on 0.4 ml of PBS, treated with 0.1 ml of a commercial IL-3 formulation containing 20 μg IL-3 (activity 2 x 10^6 units) and 0.025% HSA in PBS. The reaction mixture was mixed overnight at room temperature. Following processing, the beads were suspended in 0.5 ml of the storage buffer and kept at 4°C until used. An assay of the supernatant solution for IL-3 activity revealed 14,144 units (0.70% of the original), indicating that 99.3% of the IL-3 had been bound to the beads.

Example 21
Growth of CTLL-2 Cells Using Immobilized IL-2 (Recombinant Ala-125 Analog)

Samples of recombinant IL-2 (ala-125 analog) immobilized on 9.64 μm blue-dyed polystyrene beads; 0.93 μm blue-dyed polystyrene beads; 9.67 μm carboxylate polystyrene beads; 9.67 μm carboxylate polystyrene beads with 6-aminocaproic acid, 1,6-diaminohexane, and 1,12-diaminododecane linking arms; 65 μm carboxylate polystyrene beads with 1,12-diaminododecane linking arm; 5.29 μm amino polystyrene beads; and Sephadex® G-10 polydextran beads with 6-aminocaproic acid and 1,6-diaminohexane linking arms (see Examples 1, 2, 6, 7, 8, 9, 10, 13, 14, and 15) were examined to determine if

immobilized IL-2 would support in vitro growth of the IL-2 dependent cell line CTLL-2, a cytotoxic T-lymphocyte cell line.

The samples of beads comprising immobilized IL-2 were washed 3 times by suspension and centrifugation in a Beckman Microfuge in RPMI-1640 tissue culture medium (Whittaker M. A. Bioproducts, Inc., Walkersville, MD) containing 4% antibiotics (Fungi-Bact Solution, Irvine Scientific, Santa Anna, CA). The IL-2 immobilized beads were resuspended in RPMI-1640 medium and used for in vitro growth experiments. Aliquots of the beads were added to individual wells in a 96-well flat-bottomed tissue culture plate (Falcon #3075, Becton Dickinson & Co., Rutherford, NJ) followed by the addition of 1 x 10^4 CTLL-2 cells (an IL-2 growth dependent cell line (TIB-214) obtained from American Type Culture Collection, Rockville, MD). The Sephadex® G-10 beads with immobilized IL-2 were very irregularly shaped and settled so fast it was impossible to accurately determine a bead/cell number. Therefore, fixed volumes of freshly vortexed beads were used in the experiment. The IL-2 immobilized beads and the CTLL-2 cells were incubated for 48 hours in a 37°C incubator with a 5% CO_2 atmosphere. After 48 hours, 1 μCi of [^{3}H]-thymidine (ICN Biomedicals Inc., Irvine, CA) was added and the mixture was incubated for an additional 4 hours. The cells were collected via a Skatron cell harvester and counted in a liquid scintillation counter to determine the amount of cell growth as determined by [^{3}H]-thymidine incorporation. The results are reported in Table 3 and demonstrate that all the above-listed immobilized IL-2 combinations support CTLL-2 cell growth.

Example 22

Growth of CTLL-2 Cells Using Immobilized IL-2 (Recombinant Natural Sequence)

Recombinant natural sequence IL-2 immobilized on 9.64 μm blue-dyed polystyrene beads was examined to

determine if it would support in vitro growth of the IL-2 dependent cell line CTLL-2. Recombinant natural sequence IL-2 was immobilized on 9.64 μm beads as described in Example 5. The IL-2 immobilized beads were washed and assayed as described in Example 21. The result is reported in Table 4 and demonstrates that immobilized recombinant natural sequence IL-2 supports CTLL-2 cell growth.

Example 23

Growth of CTLL-2 Cells Using Immobilized IL-2: Carboxyl Group vs Amino Group Attachment

Recombinant IL-2 (ala-125 analog) immobilized on 9.67 μm carboxylate beads with a 1,12-diaminododecane spacer arm attached to the IL-2 via a carboxyl group was examined to determine if it supports in vitro growth of the IL-2 dependent cell line CTLL-2. Recombinant IL-2 was immobilized on 9.67 μm carboxylate beads with a 1,12-diaminododecane spacer via carboxyl groups on the IL-2 molecule as described in Example 11. The immobilized IL-2 beads were washed and assayed as described in Example 21. The growth of CTLL-2 cells using IL-2 immobilized via a carboxyl group on the IL-2 was compared to the growth of CTLL-2 cells using IL-2 immobilized via an amino group on the IL-2 (as described in Example 1). The results are reported in Table 5 and demonstrate that IL-2 attached via a carboxyl group to a bead supports CTLL-2 growth and appears more active than IL-2 attached via an amino group to a bead (see Figure 1).

Example 24

Growth of CTLL-2 Cells Using Immobilized Polyethylene Glycol Modified IL-2

Chemically modified (polyethylene glycol) recombinant IL-2 (ala-125 analog) immobilized on 9.67 μm carboxylate polystyrene beads with a 1,12-diaminododecane spacer group was examined to determine if it supports in

vitro growth of the IL-2 dependent cell line CTLL-2. IL-2 was chemically modified and immobilized according to the procedure outlined in Example 12. The immobilized chemically modified IL-2 beads were washed and assayed as described in Example 21. The results of the cell growth are shown in Table 6 and demonstrate that PEG-IL-2 beads support CTLL-2 growth.

Example 25

Concentration Dependence of Immobilized Recombinant IL-2 on Growth of CTLL-2 Cells

The effect of the concentration (units/ml or mg/ml) of recombinant IL-2 (ala-125 analog), immobilized on polystyrene beads, on the growth of CTLL-2 cells was examined. Recombinant IL-2 was immobilized on 9.64 μm blue-dyed polystyrene beads as described in Example 4. These beads were washed and assayed as described in Example 21. Concentrations of 1 and 10 beads per cell were used. Under these conditions, growth of the CTLL-2 cells was determined to be concentration dependent (see Figure 2).

Example 26

Growth of CTLL-2 Cells vs. Time Using Immobilized IL-2

The growth of CTLL-2 cells on immobilized recombinant IL-2 (ala-125 analog) was measured as a function of time and compared to the growth of CTLL-2 cells on soluble IL-2. Recombinant IL-2 was immobilized on 9.64 μm blue-dyed polystyrene beads as described in Example 1. The beads were washed as described in Example 21. Aliquots of IL-2 immobilized beads (1, 5, and 10 beads/cell) were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of 1×10^4 CTLL-2 cells (an IL-2 growth dependent cell line). The beads containing immobilized IL-2 and the CTLL-2 cells were incubated for various times in a 37°C

incubator with a 5% CO_2 atmosphere. At the end of each time period, 1 μCi of [^{3}H]-thymidine was added and the mixture was incubated for an additional 4 hours. The cells were collected using a Skatron cell harvester and counted in a liquid scintillation counter to determine cell growth. The results are graphically presented in Figure 3 along with the results of an analysis using soluble IL-2 (100 units/ml and 1000 units/ml). These results demonstrate that the growth of the CTLL-2 cells using immobilized IL-2 was comparable to or better than the growth of the CTLL-2 cells using the control, i.e., soluble IL-2. With one bead/cell, growth is not as dramatic in the 24 to 120 hour range as soluble IL-2, but growth remains steady up to 168 hours.

Example 27

Growth of CTLL-2 Cells on Recycled IL-2 Immobilized Beads

Recombinant IL-2 immobilized on 9.64 μm blue-dyed polystyrene beads was prepared as described in Example 1, and washed as described in Example 21. These IL-2 immobilized beads were tested for their ability to be reused and to maintain long term cell cultures. Aliquots of IL-2 immobilized beads were added to sterile 1.5 ml screw cap microfuge tubes (Sarstedt Inc., Princeton, NJ), inoculated with 1 x 10^4 CTLL-2 cells, and incubated for 72 hours in a 37°C incubator with 5% CO_2 atmosphere. To several of the cultures, 1 μCi of [^{3}H]-thymidine was added and the mixture was incubated for an additional 4 hours. The cells were collected via Skatron cell harvester and counted in a liquid scintillation counter to determine cell growth. The remaining cultures were centrifuged for 5 minutes in a Beckman microfuge and the supernatant was removed and discarded. These cultures were then washed 5 times with 1 ml of RPMI-1640 tissue culture medium containing 4% antibiotics, stirred by vigorous vortexing, and centrifuged (this procedure eliminates over 90% of the cells). After the fifth washing, the IL-2 immobilized

beads were resuspended in fresh medium, fresh CTLL-2 cells were added, and the 72 hour growth cycle was repeated. This procedure was repeated several times. The results are presented in Table 7, which demonstrates that IL-2 immobilized beads supported growth of CTLL-2 cells for four 72 hour growth cycles while soluble IL-2 could only support significant CTLL-2 growth for two cycles.

Example 28

Growth of Human Peripheral Blood Lymphocytes on Immobilized Recombinant IL-2

The growth of human peripheral blood lymphocytes (PBL's) on immobilized recombinant IL-2 (ala-125 analog) was examined. Recombinant IL-2 was immobilized on 9.64 μm blue-dyed polystyrene beads as described in Example 1. The immobilized IL-2 beads were prepared as described in Example 21 and used in the following experiment. PBL's were isolated from healthy donors by the following procedure. Lymphocytes were isolated from heparinized blood after centrifugation over LeucoPREP (Becton Dickinson & Co.) cell separation medium. The crude lymphocyte preparation was washed 3 times by centrifugation in RPMI-1640 tissue culture medium containing 4% antibiotics and 5% human AB serum (heat inactivated, North American Biologicals, Inc., Miami, FL). 2×10^5 PBL's were added to various concentrations of IL-2 immobilized beads. The cells were incubated for various time periods in a 37°C incubator with a 5% CO_2 atmosphere. At the end of each time period, 1 μCi of [^{3}H]-thymidine was added and the mixture was incubated for an additional 4 hours. The cells were collected via Skatron cell harvester and counted in a liquid scintillation counter to determine cell growth. The results are presented graphically in Figure 4. This example demonstrates that PBL's grow using immobilized IL-2, and that the growth of the PBL's is equal to or better than control soluble IL-2, especially after 72 hours of culture.

Example 29

LAK Cell Activity Induced by Soluble Recombinant IL-2 or Immobilized IL-2

Human PBL's grown on immobilized recombinant IL-2 (ala-125 analog) were examined to determine if they exhibit lymphokine-activated killer (LAK) cell activity. Human PBL's were isolated as described in Example 28, activated for 96 hours with IL-2 immobilized beads prepared as described in Example 1, and washed as described in Example 21. The LAK cell killing activity was assayed using the cell targets K562, Raji, and Daudi. The assay for LAK cell killing used a 4 hour ^{51}Cr release assay that has been described in the literature. See T. L. Whiteside et al., Cancer Immunol. Immunother., 26, 1 (1988); H. F. Pross et al., J. Clinical Immunology, 1, 51 (1981). Normal NK (natural killer) cells isolated from fresh PBL's killed K652 cells but did not kill Raji or Daudi cells when they were in an activated state. The results are reported in Table 8. IL-2 immobilized beads activated LAK cells killed K562, Raji, and Daudi cells. Killing was equal to soluble IL-2 activated LAK cells.

Example 30

NK/LAK Activity Induced by Immobilized IL-2

Recombinant IL-2 (ala-125 analog) immobilized on 9.64 μm blue-dyed polystyrene beads (Example 1) and 65 μm polystyrene beads (Example 10) were examined to determine if they stimulate murine lymphocytes in an *ex vivo* experiment to increase natural killing (NK) or lymphokine-activated killing (LAK) of a target cell line. That is, an *ex vivo* experiment was conducted to determine if the immobilized IL-2 beads could activate the host's immune system in the same manner that soluble IL-2 can activate LAK cell production *in vivo*. The experiment was performed as follows: Mature Balb/C male mice (groups of three, 17 weeks old) were injected i.p. with 200 μl of PBS, 50,000

units recombinant soluble IL-2, 200,000 units IL-2 immobilized on 9.64 μm blue beads (Example 1), or 100,000 units IL-2 immobilized on 65 μm beads (Example 10). After 96 hours, cells from the peritoneal cavity and spleens were collected and assayed for NK/LAK cell activity. Splenocytes were prepared from fresh spleens as described by M. H. Zaroukian et al., Immunol. Invest., 15, 813 (1986) and C. W. Gilbert et al., J. Immunol., 140, 2821 (1988). NK/LAK cell activity was assayed by a 4 hour ^{51}Cr release assay, also described in the above references. The results of the ex vivo experiment are summarized in Table 9. This data indicates that soluble IL-2 activates murine splenocytes as expected, and immobilized IL-2 on 65 μm beads also activate LAK cells in the peritoneal cavity. The LAK cell activity in the peritoneal cavity appears to be localized and may have a therapeutic value in the localized treatment of cancer.

Example 31

Growth of Human Peripheral Blood Lymphocytes on Immobilized Recombinant IL-4

Recombinant IL-4 was immobilized on 9.64 μm blue-dyed polystyrene beads as described in Example 16. The immobilized IL-4 beads were washed as described in Example 21, and used in a PHA (phytohaemagglutinin) co-stimulation experiment to induce T-cell proliferation. Peripheral blood lymphocytes were obtained from healthy donors. An enriched T-cell population was isolated from lymphocytes that were isolated from heparinized blood and separated over a Ficoll gradient (LSM, Lymphocyte Separation Medium, Organon Teknika Corp., Durham, NC). Crude lymphocytes were incubated in plastic tissue culture flasks at 37°C in RPMI-1640 medium containing 5% heat inactivated human AB serum for 1 hour to remove monocytes and other adherent cells that interfere with the costimulation T-cell proliferation assay. This step was repeated twice. Nonadherent lymphocytes, enriched with T-

cells were then used in a PHA co-stimulation proliferation assay. 1 x 10^5 cells were added to each well, in addition to soluble IL-4 (100 units/ml), PHA (0.05 μg/ml), PHA plus soluble IL-4 (100 units/ml and 1 unit/ml), or PHA plus immobilized IL-4 on beads (0.5 and 1 bead/cell starting concentration), and incubated for 96 hours at 37°C. After 96 hours, the cultures were pulsed with [^{3}H]-thymidine for 4 hours to determine T-cell proliferation. The results are listed in Table 10 and indicate that immobilized IL-4 beads stimulate T-cell proliferation over background suboptimal PHA levels.

Example 32

Growth of Human Peripheral Blood Lymphocytes on Immobilized Recombinant IL-6

Recombinant IL-6 was immobilized on 9.64 μm blue-dyed polystyrene beads as described in Example 17. The immobilized IL-6 beads were washed as described in Example 21, and used in a PHA (phytohaemagglutinin) co-stimulation experiment to induce T-cell proliferation. Peripheral blood lymphocytes were obtained from healthy donors. An enriched T-cell population was isolated from lymphocytes that were isolated from heparinized blood and separated over a Ficoll gradient (LSM, Lymphocyte Separation Medium). Crude lymphocytes were incubated in plastic tissue culture flasks at 37°C in RPMI-1640 containing 5% heat inactivated human AB serum for 1 hour to remove monocytes and other adherent cells that interfere with the costimulation T-cell proliferation assay. This step was repeated twice. Nonadherent lymphocytes, enriched with T-cells were then used in a PHA co-stimulation proliferation assay. 1 x 10^5 cells were added to each well, in addition to nothing, soluble IL-6 (100 units/ml), PHA (0.05 μg/ml), PHA plus soluble IL-6 (100 units/ml and 1 unit/ml), or PHA plus immobilized IL-6 on beads (0.5 and 1 bead/cell starting concentration), and incubated for 96 hours at 37°C. After 96 hours, the

cultures were pulsed with [^{3}H]-thymidine for 4 hours to determine T-cell proliferation. The results are tabulated in Table 11 and indicate that immobilized IL-6 beads stimulate T-cell proliferation over background suboptimal PHA levels.

Example 33
Growth of AML-193 Cells on Immobilized Recombinant Human GMCSF

Recombinant human GMCSF (rHuGMCSF) immobilized on 0.93 μm blue-dyed polystyrene beads was examined to determine if it would support in vitro growth of a GMCSF dependent cell line AML-193. Recombinant human GMCSF was immobilized on 0.93 μm blue-dyed beads as described in Example 19. The immobilized recombinant human GMCSF beads were washed as described in Example 21. The growth assay for AML-193 cell line was as follows. Aliquots of the washed beads were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of 1 x 10^4 AML-193 cells (an IL-3/GMCSF dependent cell line obtained from American Type Culture Collection, Rockville, MD). The beads with immobilized rHuGMCSF were incubated with the AML-193 cells for 116 hours in a 37°C incubator with 5% CO_2 atmosphere. After 116 hours, 1 μCi of [^{3}H]-thymidine was added and the mixture was incubated for an additional 4 hours. The cells were collected as described in Example 21. The results are reported in Table 12 and demonstrate that immobilized recombinant human GMCSF supports AML-193 cell growth.

Example 34
Growth of AML-193 Cells on Immobilized Recombinant IL-3

Recombinant IL-3 immobilized on 9.64 μm blue-dyed polystyrene beads was examined to determine if it would support in vitro growth of an IL-3/GMCSF dependent cell line AML-193. Recombinant IL-3 was immobilized on 9.64 μm blue-dyed beads as described in Example 20. The

immobilized IL-3 beads were washed as described in Example 21 and assayed as described in Example 33. The results are reported in Table 13 and demonstrate that immobilized recombinant IL-3 supports AML-193 cell growth.

Example 35
Attachment of IL-1-beta to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant IL-1-beta (Amgen) was immobilized on 9.64 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.15 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), then activated with glutaraldehyde as described in Example 1. The beads were then suspended in 0.46 ml of PBS, treated with 0.04 ml of a commercial IL-1-beta formulation containing 8.0 μg IL-1-beta (activity 4 x 10^6 units) and 0.025% HSA in PBS. The reaction mixture was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 36
Attachment of IL-1-alpha to Blue-Dyed Polystyrene Beads (9.64 μm)

Human sequence IL-1-alpha (R & D Systems) was immobilized on 9.64 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.20 ml of a 2.5% suspension of blue-dyed beads was washed with phosphate buffered saline (PBS) (pH 7.40, 3 x 1.0 ml), and then activated with glutaraldehyde as described in Example 1. The activated beads were suspended in 0.42 ml of PBS, then treated with 0.08 ml of a formulation that contained 8.0 μg of the cytokine and 200 μg human serum albumin (HSA) in PBS. The reaction mixture was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were centrifuged, washed with PBS (0.5 ml), then

treated with ethanolamine as described in Example 1. The beads were then washed (3 x 1.0 ml) with a solution containing 0.1% sodium dodecyl sulfate (SDS) in PBS in an effort to remove the last traces of any noncovalently bound cytokine. Following these washes, the beads were further processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 37

Attachment of Recombinant Human Granulocyte Colony Stimulating Factor (rHuGCSF) to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant human GCSF (rHuGCSF, Amgen) was immobilized on 9.64 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.20 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), then activated with glutaraldehyde as described in Example 1. The activated beads were suspended in 0.3 ml PBS and treated with 0.2 ml of a commercial rHuGCSF formulation containing 0.5 μg (activity 1×10^5 units) of the growth factor and 0.025% HSA in 0.01 M sodium acetate (pH 5.4). The suspension was mixed overnight at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 38

Attachment of Recombinant Murine Granulocyte Macrophage Colony Stimulating Factor (rMuGMCSF) to Blue-Dyed Polystyrene Beads (0.93 μm)

Recombinant murine GMCSF (Amgen) was immobilized on 0.93 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.25 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), then activated with 8% glutaraldehyde

as described in Examples 1 and 2. The activated beads were then suspended in 0.50 ml of a commercial rMuGMCSF formulation containing 5.0 μg (activity 5 x 10^3 units) of the growth factor and 0.025% BSA in PBS. The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Examples 1 and 2, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 39

Covalent Attachment/Adsorption of rMuGMCSF to Blue-Dyed Polystyrene Beads (0.93 μm)

The pellets obtained from two 0.2 ml-portions of a 2.5% suspension of 0.93 μm blue-dyed beads were washed with PBS (3 x 1.0 ml). One pellet (labeled C) was then activated with 1.0 ml of 8.0% glutaraldehyde in PBS for 20 hours at room temperature as described in Examples 1 and 2. The other pellet (labeled A) was suspended in 1.0 ml of PBS and also mixed for 20 hours. Both suspensions were centrifuged and the pellets washed with PBS (3 x 1.0 ml). Each pellet was then suspended in a 0.1 ml-portion of PBS and treated with 0.4 ml-portions (4.0 μg, activity 4000 units) of the commercial rMuGMCSF formulation used in Example 38. The suspensions were then mixed overnight at room temperature, centrifuged, and the supernatants removed and saved. The two pellets were again suspended in 0.5 ml- portions of PBS, centrifuged, and the supernatants removed and added to the first supernatants (labeled A1 and C1, both <u>ca</u>. 1.0 ml). The pellets were then treated with 1.0 ml- portions of 0.5 M ethanolamine as described in Example 1. The supernatants (labeled A2 and C2) were removed and saved. The pellets were then suspended in 1.0 ml-portions of PBS, centrifuged, and the supernatants (labeled A3 and C3) were removed and saved. The pellets were then suspended three times in 1.0 ml-portions of 0.1% SDS/PBS, mixed for one hour, centrifuged, and the supernatants (labeled A4, A5, A6, C4, C5, and C6,

respectively) were removed and saved. The pellets were washed with 1.0 ml-portions of PBS, and the supernatants (labeled A7 and C7) were removed and saved. The pellets were then treated with 1% BSA/PBS as described in Example 1, and the various supernatants (labeled A8, A9, A10, C8, C9, and C10, respectively) were removed and saved. The beads were finally suspended in 0.5 ml of the storage buffer and, together with the supernatants, kept at 4°C until used.

Example 40

Attachment of Recombinant Human Insulin-like Growth Factor I (rHuILGF-I) to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant human insulin-like growth factor I (rHuILGF-I, Somatomedin C, available from Bachem) was immobilized on 9.64 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml) and then activated with 1.0 ml of 8.0% glutaraldehyde in PBS as described in Example 1. The washed, activated beads were suspended in 0.42 ml PBS and treated with 0.08 ml of a solution that contained 20.0 μg of the commercial growth factor in sterile water. The suspension was mixed for 20 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 41

Attachment of Recombinant Human Insulin-like Growth Factor II (rHuILGF-II) to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant human insulin-like growth factor II (rHuILGF-II, available from Bachem) was immobilized on 9.64 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0

ml) and then activated with 1.0 ml of 8.0% glutaraldehyde/PBS as described in Example 1. The washed, activated beads were then suspended in 0.45 ml PBS and treated with 0.05 ml of a solution that contained 12.5 μg of the growth factor in sterile water. The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 42
Attachment of Recombinant Human Tumor Necrosis Factor (TNF-alpha/Cachectin) to Blue-Dyed Polystyrene Beads (9.64 μm)

Recombinant human TNF-alpha (Amgen) was immobilized on 9.64 μm blue-dyed beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then activated with glutaraldehyde as described in Example 1. Following the activation, the washed beads were suspended in 0.46 ml PBS and treated with 0.04 ml of a commercial recombinant human TNF-alpha formulation containing 19.2 μg (activity 1.92 x 10^5 units) of the growth factor in a 0.04 M Tris/0.1 M NaCl buffer (pH 8.60). The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 43
Attachment of Fibroblast Growth Factor Basic (FGFb) to Blue-Dyed Polystyrene Beads (2.85 μm)

Fibroblast Growth Factor Basic (Amgen) was immobilized on 2.85 μm blue-dyed beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then activated with glutaraldehyde as described in

Example 1. Following activation, the washed beads were suspended in 0.44 ml PBS, then treated with 0.06 ml of a commercial FGFb formulation containing 30 μg of the growth factor in a 0.02 M sodium citrate/0.1 M sodium chloride buffer (pH 5.0). The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 39, then suspended in 0.5 ml of the storage buffer, and together with the various supernatants, kept at 4°C until used.

Example 44

Attachment of Transforming Growth Factor-beta-2 (TGF-beta-2) to Blue-Dyed Polystyrene Beads (2.85 μm)

TGF-beta-2 (R & D Systems) was immobilized on 2.85 μm blue-dyed beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then treated with glutaraldehyde as described in Example 1. Following activation, the washed beads were suspended in 0.35 ml PBS, treated with 0.15 ml of a solution containing 7.5 μg of the growth factor in 0.01% Triton X-100. The suspension was mixed for 18 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 39, then suspended in 0.5 ml of the storage buffer and, together with the various supernatants, kept at 4°C until used.

Example 45

Attachment of Recombinant Human Interferon-alpha-2A (Roferon® A) to Blue-Dyed Polystyrene Beads (2.85 μm)

Recombinant human Interferon-alpha-2A (Roferon® A, Roche Laboratories) was immobilized on 2.85 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then treated with glutaraldehyde as described in Example 1. The washed, activated beads were then suspended in 0.4 ml

PBS and treated with 0.1 ml (activity 6 x 10^5 units) of a commercial recombinant human Interferon-alpha-2A aqueous formulation containing 0.9 mg sodium chloride, 0.5 mg HSA, and 0.3 mg phenol. The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 39, then suspended in 0.5 ml of the storage buffer and, together with the various supernatants, kept at 4°C until used.

Example 46

Attachment of Recombinant Human Epidermal Growth Factor (rHuEGF) to Blue-Dyed Polystyrene Beads (0.93 μm)

Recombinant human EGF (rHuEGF, available from Amgen) was immobilized on 0.93 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% slurry of blue-dyed beads was washed with PBS (3 x 1.0 ml), then treated with glutaraldehyde as described in Examples 1 and 2. The washed, activated beads were suspended in 0.35 ml PBS, then treated with 0.15 ml of a solution that contained 25.0 μg of the growth factor in PBS (pH 7.40). The suspension was mixed for 18 hours at room temperature. Following the coupling reaction, the beads were processed as described in Examples 1 and 2, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 47

Attachment of Recombinant Human Platelet-Derived Growth Factor (rHuPDGF) to Blue-Dyed Polystyrene Beads (2.85 μm)

Recombinant human PDGF (rHuPDGF, available from Bachem) was immobilized on 2.85 μm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then treated with glutaraldehyde as described in Example 1. Following activation, the washed beads were suspended in 0.35 ml PBS, and treated with 0.15 ml of a solution containing 15.0 μg of the growth factor

in sterile water. The suspension was mixed for 20 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

Example 48
Attachment of Recombinant Human Erythropoietin (rHuEPO) to Co-Bind™ Well Strips

Recombinant human erythropoietin (rHuEPO) was obtained from Amgen as a liquid formulation containing 5000 units activity per ml of a solution comprised of 50% glycerol in 0.025 M HEPES buffer (pH 7.20). The Co-Bind™ well strips, strips whose surfaces have been chemically modified (i.e., activated) to covalently bind proteins, were obtained from Micro Membranes, Inc., Newark, NJ.

Four wells of the 8-well strip were then filled as shown below:

	rHuEPO		Buffer, mls
Well	Units	mls	50% glycerol in 0.025 M HEPES, pH 7.20
A	200	0.04	0.16
B	100	0.02	0.18
C	50	0.01	0.19
D	0	0.00	0.20

The strip was covered and incubated at 35°C for 3 hours. The supernatants A-D were then removed and saved for residual activity assays. The wells were washed with buffer (2 x 0.1 ml), then treated with 0.2 ml portions of freshly prepared 1% BSA/PBS and again incubated at 35°C for one hour. These supernatants were discarded. The wells were then thoroughly washed (3 x 0.2 ml) with RPMI-7640 tissue culture medium containing 1% Fungizone, then filled with the same media. The strip was covered and kept at 4°C until used.

Example 49

Growth of CTLL-2 Cells Using IL-2 Produced From LBRM.TG6 Cells Stimulated With Immobilized Recombinant IL-1-beta Polystyrene Beads

Recombinant IL-1-beta immobilized on 9.63 μm blue-dyed polystyrene beads induces the murine lymphoma cell line LBRM.TG6 (American Type Culture Collection Co., Rockville, MD) to synthesize IL-2 which was then assayed in the IL-2 dependent CTLL-2 cell line. The immobilized IL-1-beta beads were washed three times by suspension and centrifugation as described in Example 21. IL-1-beta beads in conjunction with a suboptimal concentration of PHA [Phytoheamagglutinin P, Wellcome Foundation, Danford, England] (10 μg/ml) were added to 5 x 10^4 LBRM.TG6 cells [J. W. Larrick et al., J. Immunol. Methods, 79, 39 (1985] in 100 μl of Iscove's MEM, (Whittaker M.A. Bioproducts, Walkersville, MD) and incubated for 48 hours at 37°C in 5% CO_2. The reaction was stopped by placing the LBRM.TG6 cells at 4°C for 24 hours. Next, a 50 μl-portion of the LBRM.TG6 cell supernatant was removed and added to 50 μl of fresh CTLL-2 cells. The released soluble IL-2 was assayed to determine if it would support CTLL-2 cell growth. CTLL-2 cell growth was dependent on IL-2 concentration and was measured by the up-take, and oxidation of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) [T. Mosmann, J. Immunol Meth., 65, 55 (1983); and M.B. Hansen, S.E. Nielson and K. Berg, J. Immunol Meth., 119: 203-210(1989)] The results are reported in Table 14 and indicate that IL-1-beta beads activate the release of soluble IL-2 from LBRM.TG6 cells, and that the IL-2 released by the LBRM.TG6 cells supports growth of IL-2 dependent CTLL-2 cells.

Example 50

Recombinant IL-1-alpha Immobilized on Polystyrene Beads Induces LBRM.TG6 Cells to Produce IL-2

Recombinant IL-1-alpha immobilized on 9.64 μm blue-dyed polystyrene beads induces the murine lymphoma cell line LBRM.TG6, American Type Culture Collection, to synthesize IL-2 which was then assayed in the IL-2 dependent CTLL-2 cell line. Human sequence IL-1-alpha was immobilized on 9.64 μm blue-dyed polystyrene beads as described in Example 36. The immobilized IL-1-alpha beads were washed three times as described in Example 21. IL-1-alpha beads in conjunction with a suboptimal concentration of PHA [Phytohemagglutinin P, Wellcome Foundation, Danford, England] (10 μg/ml) were added to 5 x 10^4 LBRM.TG6 cells in 100 μl of Iscove's MEM and incubated for 48 hours at 37°C in 5% CO_2. The reaction was stopped by placing the LBRM.TG6 cells at 4°C for 24 hours. Next, 50 μl of the LBRM.TG6 cell supernatant was removed and added to 50 μl of fresh CTLL-2 cells. The released soluble IL-2 was assayed as described in Example 49. The results are reported in Table 15 and indicate that IL-1-alpha beads activate the release of soluble IL-2 from LBRM.TG6 cells, and that the IL-2 released by the LBRM.TG6 cells supports growth of IL-2 dependent CTLL-2 cells.

Example 51

Growth of AML-193 Cells on Immobilized Recombinant Human GCSF

Recombinant human GCSF (rHuGCSF) immobilized on 9.64 μm blue-dyed polystyrene beads was examined to determine if it would support *in vitro* growth of a growth factor (GCSF) dependent cell line AML-193, American Type Culture Collection. Recombinant human GCSF was immobilized on 9.64 μm blue-dyed polystyrene beads as described in Example 37. The immobilized rHuGCSF beads

were washed as described in Example 21. The growth assay for AML-193 cell line was as follows. Aliquots of the washed beads were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of 1 x 10^4 AML-193 cells as in Example 33. The beads with the immobilized rHuGCSF were incubated with the AML-193 cells for 116 hours in a 37°C incubator with a 5% CO_2 atmosphere. [^{3}H]-thymidine (1 μCi) was then added to each well and the mixture was incubated for an additional 4 hours. The cells were collected as described in Example 21. The results are reported in Table 16 and demonstrate that immobilized recombinant human GCSF supports AML-193 cell growth.

Example 52

Recombinant Murine GMCSF (rMuGMCSF) Immobilized on 0.93 μm Polystyrene Beads Stimulates Granulopoiesis in BDF1 Mice

Recombinant murine GMCSF (rMuGMCSF) immobilized on 0.93 μm blue-dyed polystyrene beads stimulates granulopoiesis in BDF1 mice. Recombinant murine GMCSF was immobilized on 0.93 μm blue-dyed polystyrene beads as described in Example 38. Immobilized rMuGMCSF beads, like soluble rMuGMCSF, stimulates granulopoiesis in the peripheral blood of mice after an injection. Ishida et al., Acta. Haemat., 8, 1 (1988) recently reported that GMCSF stimulates granulopoiesis in the peripheral blood of mice after a single injection of soluble GMCSF. These experiments were repeated using immobilized rMuGMCSF to determine if immobilized rMuGMCSF is active in vivo. BDF1 mice were injected with either soluble rMuGMCSF (20 units i.p.) or immobilized rMuGMCSF (50 units i.v.). Peripheral blood was drawn from the retro-orbital sinus of BDF1 mice at 0, 6, 12, 24, 48, 72, and 96 hours and the number of neutrophils (PMN)/ml was determined from a complete blood count. The results are shown in Figure 5 and indicate that immobilized rMuGMCSF is active in vivo. Furthermore, the results indicate that the beads stimulate PMN

production in numbers (about a 2-fold increase) and rates (maximum in 12 hours with decline to initial levels within 24 to 48 hours) comparable to soluble rMuGMCSF.

Example 53

Recombinant Murine GMCSF (rMuGMCSF) Immobilized on 0.93 μm Polystyrene Beads Stimulates Granulopoiesis in Cyclophosphamide-Treated BDF1 Mice

Recombinant murine GMCSF (rMuGMCSF) immobilized on 0.93 μm blue-dyed polystyrene beads stimulates granulopoiesis in cyclophosphamide-treated mice. Recombinant murine GMCSF was immobilized on 0.93 μm blue-dyed polystyrene beads as described in Example 38. Immobilized rMuGMCSF beads like soluble rMuGMCSF stimulates granulopoiesis in the peripheral blood of mice after an injection. Ishida et al., Acta. Haemat., 8, 1 (1988) recently reported that GMCSF stimulates granulopoiesis in the peripheral blood of mice after their lymphocyte population was depleted by a single injection of cyclophosphamide. Repeated doses of GMCSF help these mice to recover lymphocyte numbers 2 to 3 days faster than untreated mice. Since immobilized rMuGMCSF shows *in vivo* activity (Example 52), cyclophosphamide-treated mice were given either soluble rMuGMCSF or immobilized rMuGMCSF to determine the effects rMuGMCSF on neutrophil counts. The experimental protocol was as follows. BDF1 mice were injected with cyclophosphamide (250 mg/Kg weight) at zero time to deplete the neutrophil cell count. Twenty-four hours later, either soluble rMuGMCSF (2 units injected i.p. every 12 hours for 6 days; or 2 units injected i.v. on days 1, 3, and 5), or immobilized rMuGMCSF (2 units injected i.v. on days 1, 3, and 5) was administered. Peripheral blood was drawn from the retro-orbital sinus of BDF1 mice at day 0, 3, 5, 7, and 9 and the number of neutrophils (PMN)/ml was determined from a complete blood count. The results are shown in Figure 6 and indicate that immobilized rMuGMCSF is active *in vivo*. Furthermore,

rMuGMCSF beads stimulate PMN production in numbers and rates comparable to soluble rMuGMCSF.

Example 54

Covalently Linked rMuGMCSF Polystyrene Beads Retain Cytokine Activity While Adsorbed rMuGMCSF Polystyrene Beads Do Not Retain Cytokine Activity

Recombinant murine GMCSF (rMuGMCSF) covalently attached to 0.93 μm blue-dyed polystyrene beads retain biological activity (i.e., promote the growth of DA1-E5 cells) while rMuGMCSF adsorbed to 0.93 μm blue-dyed polystyrene beads do not retain biological activity (i.e., DA1-E5 cells do not grow). Covalent and adsorbed rMuGMCSF blue-dyed polystyrene beads were prepared and washed as described in Example 39. Beads were washed three times before the assays described in Example 21 were performed. DA1-E5 cells an IL-3/GMCSF/EPO dependent cell line, obtained from Dr. Larry Gilbert, University of Alta, Edmonton, Alberta, Canada, were used to assay both soluble fractions of rMuGMCSF and immobilized rMuGMCSF bead fractions (covalently bound or adsorbed). The rMuGMCSF assay is as follows. DA1-E5 cells ($1x10^4$) were incubated with either soluble rMuGMCSF or immobilized rMuGMCSF (covalent or adsorbed) for 48 hours as described in Example 21. Either MTT or 1 μCi of [^{3}H]-thymidine was added. The mixture was incubated for an additional 4 hours. Cells were harvested as described in Example 21. When the polystyrene beads were washed with sodium dodecyl sulfate (SDS), the adsorbed rMuGMCSF was removed (Figure 7). These beads no longer retained any biological activity. Covalently linked rMuGMCSF, however, did not wash off with SDS. These beads retained biological activity. The results are listed in Table 17.

Example 55

Recombinant Human Insulin-Like Growth Factor-I (rHuILGF-I) Immobilized on 9.63 μm Blue-dyed Polystyrene Beads Stimulates a Crude Lymphocytic Preparation to Proliferate in Serum-Free Medium

Recombinant human ILGF-I was immobilized on 9.63 μm blue-dyed polystyrene beads as described in Example 40. Immobilized rHuILGF-I beads were washed as described in Example 21. Schimpff et al., Acta Endocrinologica, 102, 21 (1983) disclose that ILGF-I in conjunction with lectin co-stimulation can induce lymphocytes to grow in serum-free medium. Recombinant human ILGF-I activity was assayed as follows: 1×10^5 lymphocytes were added to individual wells of 96-well flat-bottomed tissue culture plates containing 100 μl of RPMI-1640 tissue culture medium, 5 μg/ml PHA, 0.25% low endotoxin BSA and either soluble rHuILGF-I or immobilized rHuILGF-I beads. The mixture was incubated for 48 hours at 37°C. Next 1 μCi/well of [^{3}H]-thymidine was added and the mixture was incubated for another 18 hours. The cells were harvested as described in Example 21. The results are summarized in Table 18. They indicate that immobilized rHuILGF-1 on polystyrene beads induce lymphocyte growth in a PHA co-stimulation assay in serum-free medium.

Example 56

Recombinant Human Insulin-Like Growth Factor II (rHuILGF-II) Immobilized on 9.63 μm Blue-Dyed Polystyrene Beads Stimulates a Crude Lymphocyte Preparation to Proliferate in Serum-Free Medium

Recombinant human ILGF-II was immobilized on 9.63 μm blue-dyed polystyrene beads as described in Example 41. The immobilized rHuILGF-II beads were washed as described in Example 21. The assay performed was as described in Example 55. Results are summarized in Table 19 and show that immobilized rHuILGF-II beads induce lymphocyte growth in a PHA co-stimulation assay in serum-free medium.

Example 57

Immobilized Recombinant Human Tumor Necrosis Factor (TNF-alpha) Kills Murine LM Cells

Recombinant tumor necrosis factor alpha (TNF-alpha) immobilized on 9.64 μm blue-dyed polystyrene beads kill murine LM cells in a 72 hour killing assay. Recombinant TNF-alpha was immobilized on 9.64 μm blue-dyed polystyrene beads as described in Example 42. The immobilized TNF-alpha beads were washed three times as described in Example 21. TNF-alpha killing was assayed using murine LM cells (American Type Culture Collection). The assay was as follows. Aliquots of either soluble TNF-alpha or immobilized TNF-alpha were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of 1×10^4 LM cells. The mixtures were incubated for 72 hours. Killing was then assayed by either the addition of 1 μCi of [^{3}H]-thymidine to each well or MTT and the mixture incubated an additional 4 hours. The thymidine-labeled cells were collected as described in Example 21. The MTT-labeled cells were fixed in isopropyl alcohol and the amount of MTT uptake measured by reading the absorbance at 590 nm. The results are reported in Table 20 and demonstrate that immobilized TNF-alpha inhibits both thymidine uptake, MTT uptake, and oxidation, which indicates cell death.

Example 58

Fibroblast Growth Factor Basic (FGFb) Immobilized on 2.85 μm Polystyrene Beads Induces Growth of Murine 3T3 Cells in Growth Factor Depleted Medium

Immobilized FGFb stimulates growth of Murine 3T3 cells in growth factor depleted medium. Immobilized FGFb beads prepared according to Example 43 were washed three times by suspension and centrifugation as described in Example 21. Murine 3T3 cells (American Type Culture Collection) were grown in 1,2-dimethoxyethane (DME) medium

with antibiotics and 10% calf serum (CS) as described by Gospodarowcz, Nature, 249, 123 (1974), incorporated herein by reference. 3T3 cells were isolated by trypsinization and plated at either 600 or 2000 cells/well (96-well plates) in DME medium plus 10% CS. 3T3 cells were incubated over night at 37°C. The next morning, the wells were washed three times, resuspended in DME medium containing 0.4% CS, and incubated an additional 24 hours to deplete the cells and medium of growth factors. After 24 hours in DME medium containing 0.4% CS, either soluble FGFb, immobilized FGFb, or 10% CS was added to individual wells and the 3T3 cells incubated an additional 24 to 48 hours. The cells were then labeled with 1 μCi/well of [^{3}H]-thymidine and incubated for an additional 16 hours. The results are displayed in Table 21. They indicate that immobilized FGFb beads stimulated 3T3 cell growth to levels comparable to soluble FGFb.

Example 59

Transforming Growth Factor-beta-2 (TGF-beta-2) Immobilized on 2.85 μm Polystyrene Beads Induces Growth of NRK-49F Cells in Growth Factor Depleted Medium

Immobilized TGF-beta-2 stimulates the growth of NRK-49F cells in growth factor depleted medium. Immobilized TGF-beta-2 beads prepared according to the method in Example 44 were washed three times by suspension and centrifugation as described in Example 21. NRK-49F cells (American Type Culture Collection) were grown in DME medium with antibiotics and 10% calf serum (CS) as described by Assoin et al., J. Biol. Chem., 258, 7155 (1973), incorporated herein by reference. NRK-49F cells were isolated by trypsinization and plated at a concentration of 5 x 10^3 cells/well (96-well plates) in DME plus 10% CS medium. The cells were incubated over night at 37°C in 5% CO_2, and then washed twice in DME medium containing 0.2% CS. The medium was replaced with 100 μl DME plus 0.2% CS and the cells were incubated as above for

three to four days to deplete the medium of growth factors. When the NRK-49F cells had reached about 75% confluency, soluble TGF-beta-2a, immobilized TGF-beta-2a, or 10% CS was added to individual wells and the NRK-49F cells were incubated an additional 17 hours. Then 1 μCi of [^{3}H]-thymidine was added to the wells and the cells were incubated for another 4 hours before harvesting as described in Example 21. The results are listed in Table 22. They indicate that immobilized TGF-beta-2 beads stimulate NRK-49F cells to grow in growth factor depleted medium.

Example 60

Immobilized Recombinant Human Interferon-alpha-2a Kills the Interferon Sensitive HeLa S3 Cell Line

Immobilized recombinant Human Interferon-alpha-2A kills the interferon sensitive HeLa S3 cell line. Recombinant human Interferon-alpha-2a (INF-alpha-2a) immobilized on 2.85 μm blue-dyed polystyrene beads inhibits [^{3}H]-thymidine uptake in a human epitheloid carcinoma cell line HeLa S3 (i.e., kills HeLa S3). Recombinant INF-alpha-2a was immobilized on 2.85 μm blue-dyed polystyrene beads as described in Example 45. The immobilized INF-alpha-2a beads were washed three times as described in Example 21. INF-alpha-2a killing was assayed using a human epithelioid carcinoma cell line HeLa S3 (American Type Culture Collection). INF-alpha-2a blocks [^{3}H]-thymidine uptake which leads to cell death. The assay was as follows. Aliquots of either soluble INF-alpha-2a or immobilized INF-alpha-2a were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of 1 x 10^4 HeLa S3 cells. The beads with the INF-alpha-2a or soluble INF-alpha-2a were incubated for either 48, or 72 hours, at which time 1 μCi of [^{3}H]--thymidine was added to each well and the mixture incubated an additional 4 hours. The cells were collected as described in Example 21. The results are reported in

Table 23 and demonstrate that immobilized INF-alpha-2a inhibits thymidine uptake which leads to the death of the HeLa S3 tumor cells.

Example 61

Recombinant Human Epidermal Growth Factor Immobilized on 0.93 μm Polystyrene Beads Induces NRK-49F Cells to Grow in the Absence of Serum

Recombinant human epidermal growth factor (rHuEGF) immobilized on 0.93 μm blue-dyed polystyrene beads induces NRK-49F cells to grow in the absence of serum. Recombinant human EGF was immobilized on 0.93 μm blue-dyed polystyrene beads as described in Example 46. The immobilized rHuEGF beads were washed three times as described in Experiment 21. Serum contains many growth factors that are required by cells to grow _in vitro_. The assay procedure for the NRK-49F cells was as follows. NRK-49F cells were maintained in DMEM (Dulbecco's Modified Eagles Medium, Whittakar M.A. Bioproducts) medium plus 10% calf serum (CS). NRK-49F cells are plated at 5×10^3 cells per well in 96-well flat-bottomed tissue culture plates and incubated for 24 hours in the 10% CS. The cells were then washed with serum-free medium and then replenished with serum-free DMEM. Aliquots of either soluble rHuEGF or immobilized rHuEGF were added to the individual wells. The beads with the rHuEGF or soluble rHuEGF were incubated for 24 or 48 hours. Growth was then measured by the addition of 1 μCi of $[^3H]$-thymidine to each well and the mixture was incubated an additional 6 hours. The cells were collected as described in Example 21. The results are reported in Table 24 and demonstrate that immobilized rHuEGF will induce murine NRK-49F cells to grow.

Example 62

Recombinant Platelet-Derived Growth Factor Immobilized on 2.85 μm Polystyrene Beads Induces Murine 3T3 Cells to Grow in the Absence of Serum

Recombinant platelet-derived growth factor (rHuPDGF) immobilized on 2.85 μm blue-dyed polystyrene beads induce murine 3T3 cells to grow in the absence of serum. Recombinant human PDGF was immobilized on 2.87 μm blue-dyed polystyrene beads as described in Example 47. The immobilized rHuPDGF beads were washed three times as described in Experiment 21. Serum contains many growth factors that are required by cells to grow <u>in</u> <u>vitro</u>. Most cells will not grow if they are depleted of these growth factors. Murine Swiss 3T3 is such a cell line, which is available from American Type Culture Collection. The assay procedure was as follows. Swiss 3T3 cells were maintained in DMEM medium plus 10% calf serum (CS). The 3T3 cells are plated at 1 x 10^4 cells per well in 96-well flat-bottomed tissue culture plates and grown to confluency. The medium was then changed to 2% CS, and the 3T3 cells remained viable but did not grow. Before growth factors were added, the cells were washed free of the 2% CS with serum-free DMEM, and then replenished with serum-free DMEM. Aliquots of either soluble rHuPDGF or immobilized rHuPDGF were added to the individual wells. The cells were incubated for 16 hours. Growth was measured by the addition of 1 μCi of [^{3}H]-thymidine to each well and the mixture was incubated an additional 6 hours. The cells were collected as described in Example 21. The results are reported in Table 25 and demonstrate that immobilized rHuPDGF will induce murine 3T3 cells to grow.

Example 63

Growth of DA1-E5 Cells on Recombinant Human Erythropoietin Immobilized on Co-Bind™ Polystyrene Plates

Recombinant human erythropoietin (rHuEPO) immobilized on Co-Bind™ polystyrene plates induces growth

of EPO/IL-3 dependent DA1-E5 cells (see Example 54). Recombinant human EPO was immobilized on Co-Bind™ polystyrene plates as described in Example 48. Wells containing Immobilized rHuEPO were washed five times with 1X PBS, followed by washing five times with Iscove's MEM containing 10% heat-inactivated serum, then filled with 0.050 ml of IMDM with 10% serum. DA1-E5 cell growth was assayed as follows. 1×10^4 cells were added to wells containing immobilized rHuEPO or soluble rHuEPO. The cells were incubated for 48 hours before either MTT or 1 μCi [^{3}H]-thymidine was added to each well and the mixture was then incubated an additional 4 hours. The results are reported in Table 26 and demonstrate that immobilized rHuEPO will induce growth in EPO/IL-3 dependent DA1-E5 cells.

Example 64

Attachment of Recombinant Human Gamma-Interferon to Co-Bind™ Well Strips

Recombinant human gamma-interferon (rHuIFN-gamma) was obtained from Genzyme, Boston, MA, as a liquid formulation that contained 1×10^6 U/ml (2.5×10^7 U/mg). An aliquot (0.02 mls, 2×10^4 U) of this solution was diluted to 2.0 ml with PBS to give a stock solution that was 1×10^4 U/ml. Four wells of the 8-well strip were then filled as shown below.

	rHuIFN-gamma		PBS, mls
Well	mls	units	
A	0.1	1000	0.1
B	0.05	500	0.15
C	0.01	100	0.19
D	0.005	50	0.195

After filling the wells, the strips were covered and incubated at 37°C for 3 hours, then processed exactly as described in Example 48. After washing thoroughly with PBS, the wells were filled with PBS, the strips were

covered and kept at 4°C until used.

Example 65

Biological Activity of Recombinant Human Gamma-Interferon

Human peripheral blood monocytes were isolated from blood drawn into a heparinized syringe and isolated by gradient centrifugation on 46% Percoll (Pharmacia, Newark, NJ). The monocytes were isolated from the interface, washed three times in phosphate buffered saline and resuspended in RPMI-1640 media containing 5% human AB sera to a concentration of 1 x 10^6 cells per ml. Co-bind™ strips containing 4 wells gamma-interferon immobilized as in Example 67 were washed three times with phosphate buffered saline, washed three times with RPMI-1640 media containing 2% Fungi-Bact, and wiped with a sterile gauze. To each well was added 1 x 10^5 monocytes in a volume of 0.1 ml. Soluble gamma-interferon was added to wells which did not contain the immobilized gamma-interferon. The cultures were incubated for 24 hours after which 0.1 ml of the media was removed and assayed for tumor necrosis factor production using commercially available Elisa kits. The results shown in Table 27 demonstrate that immobilized gamma-interferon is biologically active.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

Table 1

Growth Factor Families

Family	Members
Epidermoid Growth Factors (EGF)	Epidermoid Growth Factor Transformation Growth Factor Alpha (TGF-alpha) Vaccinia Growth Factor (VGF) Shape Fibroma Growth Factor (SFGF) Myxoma Growth Factor (MGF) Amphiregulin (AR)
Platelet-Derived Growth Factor (PDGF)	PDGF-AA PDGF-AB PDGF-BB
Transformation Growth Factor Beta (TGF-beta)	TGF-beta 1 TGF-beta 2 TGF-beta 3 TGF-beta 4 Inhibins Mulierian Inhibiting Substance (MIS) Bone Morphogenic Proteins (BMP's)
Fibroblast Growth Factor (FGF)	Acidic FGF Basic FGF hst gene product int-2 gene product
Insulin-like Growth Factor (IGF)	IGF-I IGF-II Insulin Relaxin

Table 2

Attachment of IL-2 to Polystyrene Blue-Dyed Beads: Effect of IL-2 Solution Concentration

Beads 2.5% Suspension µl	PBS µl	IL-2 Solution µl	IL-2 Solution µg	Activity (units)* IL-2 Solution before	Activity (units)* IL-2 Solution after	% IL-2 Incorporated
125	250.0	0	0	----	----	----
125	247.5	2.5	2.5	18,750	4	100.0
125	245.0	5.0	5.0	37,500	6	100.0
125	240.0	10.0	10.0	75,000	1,678	97.8
125	225.0	25.0	25.0	187,500	5,450	97.1
125	200.0	50.0	50.0	375,000	27,140	92.8
125	100.0	100.0	100.0	750,000	82,216	89.1
125	50.0	200.0	200.0	1,500,000	377,767	74.8

* Activity of the IL-2 solution before and recovered from supernatant after attachment of the IL-2 to the beads

Table 3

Growth of CTLL-2 Cells Using Immobilized Recombinant IL-2 (Ala-125 Analog)

Example No.	Recombinant IL-2 Type	$[^3H]$-Thymidine Incorporation (DPM's)	Concentration of IL-2 Bead (units/Beads)	Total IL-2 Activity (units/ reaction)	Relative Activity Compared Soluble IL-2 (1000 units/ml)
---	Soluble IL-2[1] (Control)	72,042 ± 9574	---	100 units	100%
1	9.64 µm Blue-dyed Beads[3] with Glutaraldehyde Linking Arm	90,975 ± 2855	0.030	3,000 units	126%
2	0.93 µm Blue-dyed Beads[2] with Glutaraldehyde Linking Arm	101,005 ± 8005	0.00046	4,600 units	140%
6	9.67 µm Carboxylate Beads[3]	32,708 ± 3108	0.027	2,700 units	45%
7	9.67 µm Carboxylate Beads[3] with 6-Aminocaproic Acid Linking Arm	53,827 ± 8439	0.027	2,700 units	75%
8	9.67 µm Carboxylate Beads[3] with 1,6-Diaminohexane/ Glutaraldehyde Linking Arm	66,319 ± 505	0.026	2,600 units	92%
9	9.67 µm Carboxylate Beads[3] with 1,12-Diaminododecane/ Glutaraldehyde Linking Arm	177,510 ± 14,175	0.047	4,720 units	246%

Table 3 (continued)

---	Soluble IL-2[1] (Control)	109,900 ± 10,550	---	100 units	100%
13	5.29 μm Amino Beads[3] with Glutaraldehyde Linking Arm	102,749 ± 10,007	0.026	2,600 units	93%
---	Soluble IL-2[1] (Control)	134,190 ± 15,700	---	100 units	100%
10	65 μm Carboxylate Beads[4] with 1,12-Diaminododecane/ Glutaraldehyde Linking Arm	197,633 ± 1585	7.2	7,200 units	147%
---	Soluble IL-2[1] (Control)	147,494 ± 32,083	---	100 units	100%
14	Sephadex® G-10 Beads[5] with 6-Aminocaproic Acid Linking Arm	195,328 ± 22,345	~45.0	9,000 units	132%
15	Sephadex® G-10 Beads[5] with 1,6-Diaminohexane Linking Arm	41,116 ± 976	~43.2	8,640 units	28%

1. IL-2 concentration was 1000 units/ml
2. 1000 beads/cell starting concentration
3. 10 beads/cell starting concentration (0.03 units IL-2/bead)
4. 0.1 beads/cell starting concentration (7.2 units IL-2/bead)
5. 10 μl of immobilized IL-2 Sephadex® G-10 beads/well (approximately 150-200 beads)

Table 4

Growth of CTLL-2 Cells Using Recombinant Natural Sequence IL-2

Example No.	Recombinant IL-2 Type	[^{3}H]-Thymidine Incorporation (DPM's)	Concentration of IL-2 Bead (units/Beads)	Total IL-2 Activity (units/reaction)	Relative Activity Compared Soluble IL-2 (1000 units/ml)
---	Soluble IL-2[1]	97,489 ± 9847	---	100 units	100%
5	IL-2 Immobilized on 9.64 µm Blue-dyed Beads[2] with Glutaraldehyde Linking Arm	36,734 ± 3734	0.005	500 units	38%

1. Soluble IL-2 concentration was 1000 units/ml
2. 10 beads/cell starting concentration (0.005 units IL-2/bead)

Table 5

Growth of CTLL-2 Cells Using Immobilized Recombinant IL-2 Polystyrene Beads: Carboxyl Group vs Amino Group Linkage

Example No.	Recombinant IL-2 Type	[^{3}H]-Thymidine Incorporation (DPM's)	Concentration of IL-2 Bead (units/Beads)	Total IL-2 Activity (units/reaction)	Relative Activity Compared Soluble IL-2 (1000 units/ml)
---	Soluble IL-2[1]	150,300	---	100 units	100%
11	9.67 μm Carboxylate Beads[2] with 1,12-Diaminododecane Linking Arm Attached to IL-2 via a Carboxyl Group	274,665	0.040	4,000 units	183%
1	9.63 μm Blue-dyed Beads with Glutaraldehyde Linking Arm Attached[3] to IL-2 via an Amino Group	182,337	0.055	5,500 units	121%

1. Soluble IL-2 concentration was 1000 units/ml
2. 10 beads/cell starting concentration (0.04 units/bead)
3. 10 beads/cell starting concentration (0.055 units/bead)

Table 6

Growth of CTLL-2 Cells Using PEG-IL-2 Immobilized on Polystyrene Beads

Example No.	Recombinant IL-2 Type	[^{3}H]-Thymidine Incorporation (DPM's)	Concentration of IL-2 Bead (units/Beads)	Total IL-2 Activity (units/reaction)	Relative Activity Compared Soluble IL-2 (1000 units/ml)
---	Soluble IL-2[1]	212,054 ± 19,556	---	100 units	100%
12	9.67 μm Carboxylate Beads[2] with 1,12-Diaminododecane Linking Arm Attached to PEG-IL-2 via a Carboxyl Group	327,113 ± 14,379	0.023	2,300 units	154%

1. Soluble IL-2 concentration was 1000 units/ml
2. 10 beads/cell starting concentration (0.023 units/bead)

Table 7

CTLL-2 Growth On Recycled IL-2 Immobilized Beads

72 Hour Growth Cycle	IL-2 Immobilized Beads (3000 units IL-2)		Soluble IL-2 (1000 units IL-2)	
	% of the Control	DPM's sample[1]/control	% of the Control	DPM's sample[1]/control
1	100%	10376	100%	12618
2	93%	13014/13995	40%	6498/16330
3[(2)]	87%	3160/3628	8%	565/7344
4	79%	15585/19657	----	----

1. Control is fresh IL-2 immobilized beads or soluble IL-2 set up at each new cycle
2. All counts were low in cycle 3

Table 8

LAK Cell Activity Induced by Soluble Recombinant IL-2 or IL-2 Immobilized Beads

Cell Type	Treatment	Lytic Units[1]		
		K562	Raji	Daudi
Plastic Nonadherent Lymphocytes	None	6	0	0
	Soluble IL-2 (100 units/ml)	53	22	80
	IL-2 Immobilized Beads (1 bead/cell)	68	22	71
Total PBL's	None	4	0	0
	Soluble IL-2 (100 units/ml)	90	22	62
	IL-2 Immobilized Beads (1 bead/cell)	76	20	65

1. 1LU (lytic unit) = 20% killing with 1 x 10^6 LAK cells

Table 9

NK/LAK Cell Killing of the Murine Target YAC-1 by *In Vivo* IL-2 Stimulated Lymphocytes from Spleen and Peritoneal Exuldates

Peritoneal Lymphocytes

Effector Target Ratio	Control (200 µl PBS)	50,000 units Soluble IL-2	9.64 µm beads 200,000 units IL-2	65 µm beads 100,000 units IL-2
	Percent Lysis			
2:1	1%	1%	1%	2%
5:1	2%	1%	0%	3%
10:1	2%	1%	2%	3%
25:1	3%	2%	1%	9%

Spleen Lymphocytes

Effector Target Ratio	Control (200 µl PBS)	50,000 units Soluble IL-2	9.64 µm beads 200,000 units IL-2	65 µm beads 100,000 units IL-2
	Percent Lysis			
2:1	1%	4%	0%	0%
5:1	1%	5%	2%	0%
10:1	2%	7%	2%	0%
25:1	5%	16%	5%	4%

Table 10

Growth of Crude T-Cells Using IL-4 Immobilized Beads and Suboptimal PHA Concentrations

Donor 1

Experimental Conditions	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control
Nothing Added	338 ± 333	----
Soluble IL-4 (100 units/ml)	439 ± 263	----
Control PHA (0.05 µg/ml)	54,831 ± 12,406	100%
PHA (0.05 µg/ml) plus Soluble IL-4 (100 units/ml)	100,555 ± 30,822	183%
PHA (0.05 µg/ml) plus Soluble IL-4 (1 unit/ml)	65,127 ± 15,470	119%
PHA (0.05 µg/ml) plus Immobilized IL-4 (0.5 beads/cell)	59,602 ± 10,210	109%
PHA (0.05 µg/ml) plus Immobilized IL-4 (1 bead/cell)	67,350 ± 7,766	123%

Table 10 (continued)

Donor 2		
Experimental Conditions	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control
Nothing Added	230 ± 231	----
Soluble IL-4 (100 units/ml)	385 ± 106	----
Control PHA (0.05 µg/ml)	5,216 ± 1,215	100%
PHA (0.05 µg/ml) plus Soluble IL-4 (100 units/ml)	31,111 ± 5,552	596%
PHA (0.05 µg/ml) plus Soluble IL-4 (1 unit/ml)	15,042 ± 4,653	288%
PHA (0.05 µg/ml) plus Immobilized IL-4 (0.5 beads/cell)	8,421 ± 1,627	161%
PHA (0.05 µg/ml) plus Immobilized IL-4 (1 bead/cell)	6,103 ± 2,201	117%

Table 11

Growth of Crude T-Cells Using IL-6 Immobilized Beads and Suboptimal PHA Concentrations

Donor 1		
Experimental Conditions	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control
Nothing Added	351 ± 333	----
Soluble IL-6 (100 units/ml)	175 ± 263	----
Control PHA (0.05 μg/ml)	55,385 ± 15,928	100%
PHA (0.05 μg/ml) plus Soluble IL-6 (100 units/ml)	57,293 ± 19,551	103%
PHA (0.05 μg/ml) plus Soluble IL-6 (1 unit/ml)	41,163 ± 13,459	74%
PHA (0.05 μg/ml) plus Immobilized IL-6 (0.5 beads/cell)	98,996 ± 23,073	179%
PHA (0.05 μg/ml) plus Immobilized IL-6 (1 bead/cell)	98,865 ± 9,654	179%

Table II (continued)

Donor 2

Experimental Conditions	[^{3}H]-Thymidine Incorporation (DPM's)	% of the Control
Nothing Added	225 ± 220	----
Soluble IL-6 (100 units/ml)	136 ± 57	----
Control PHA (0.05 µg/ml)	5,022 ± 1,780	100%
PHA (0.05 µg/ml) plus Soluble IL-6 (100 units/ml)	6,163 ± 1,624	123%
PHA (0.05 µg/ml) plus Soluble IL-6 (1 unit/ml)	3,946 ± 808	79%
PHA (0.05 µg/ml) plus Immobilized IL-6 (0.5 beads/cell)	33,074 ± 6,697	659%
PHA (0.05 µg/ml) plus Immobilized IL-6 (1 bead/cell)	37,408 ± 7,103	745%

Table 12

Growth of AML-193 Cells on Immobilized Recombinant Human GMCSF Polystyrene Beads

Example No.	Recombinant Human GMCSF (rHuGMCSF)	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control
---	Soluble GMCSF[1] (Control)	40,952 ± 1665	100%
33	rHuGMCSF Immobilized on 0.93 μm Blue-dyed Beads[2] with Glutaraldehyde Linking Arm	44,120 ± 9593	108%
33	rHuGMCSF Immobilized 0.93 μm Blue-dyed Beads[3] with Glutaraldehyde Linking Arm	51,586 ± 927	126%

1. Soluble recombinant human GMCSF concentration was 100 units/ml
2. 1.8 x 10^3 beads/cell initial concentration (6.67 x 10^{-6} units/bead)
3. 1.8 x 10^4 beads/cell initial concentration

Table 13

Growth of AML-193 Cells Using Immobilized Recombinant IL-3 Polystyrene Beads

Example No.	Recombinant IL-3	[^{3}H]-Thymidine Incorporation (DPM's)	% of the Control
---	Soluble IL-3[1] (Control)	20,517 ± 1169	100%
34	IL-3 Immobilized on 9.64 µm Blue-dyed Beads[2] with Glutaraldehyde Linking Arm	23,271 ± 1396	113%

1. Soluble IL-3 concentration was 1000 units/ml
2. 10 beads/cell starting concentration (0.15 units/bead)

Table 14

Growth of CTLL-2 Cells Using IL-2 Produced From LBRM.TG6 Cells Stimulated with Immobilized Recombinant IL-1-beta Polystyrene Beads

	MTT Incorporation Absorption at 590nm (A_{590})	% of the Control
Control (10 μg PHA/ml)	0.051 ± 0.003	0%
Soluble IL-2 (10 units/ml)	0.122 ± 0.003	100%
Soluble IL-1-beta (10 units/ml)	0.069 ± 0.008	25%
Soluble IL-1-beta (100 units/ml)	0.125 -----	104%
0.20 Beads/Cell (80 units Immobilized IL-1-beta)	0.098 ± 0.006	66%
1.00 Beads/Cell (400 units Immobilized IL-1beta)	0.123 ± 0.003	101%
1.00 Beads/Cell (Blank Beads containing BSA)	0.045 ± 0.001	0%

Table 15

Growth of CTLL-2 Cells Using IL-2 Produced from LBRM.TG6 Cells Stimulated with Immobilized Human IL-1-alpha Polystyrene Beads

Conditions	MTT Incorporation Absorption 590 nm	% of the Control
Control	0.116 ± 0.009	0%
<u>Soluble IL-1-alpha</u>		
10 units/ml	0.117 ± 0.007	13%
100 units/ml	0.124 ± 0.014	100%
<u>Immobilized IL-1-alpha</u>		
16 units/ml	0.130 ± 0.006	175%
80 units/ml	0.209 ± 0.005	1163%
160 units/ml	0.174 ± 0.010	725%

Specific Activity = 0.0008 ng/bead

Table 16

Growth of AML-193 Cells Using Immobilized Recombinant Human GCSF (rHuGCSF) Polystyrene Beads

Conditions	[^{3}H]-Thymidine Incorporation (DPM'S)	% of the Control
Soluble rHuGCSF		
Control (none)	5713 ± 1702	0%
10 units/ml	11000	60%
50 units/ml	14525 ± 2595	100%
Immobilized rHuGCSF Beads		
Control (no beads)	4552 ± 832	-13%
0.5 Beads/cell (10 units/ml)	7363 ± 1710	19%
1.0 Beads/cell (20 units/ml)	8244 ± 2031	29%
2.0 Beads/cell (40 units/ml)	9503 ± 906	43%
5.0 Beads/cell (100 units/cell)	10098 ± 1142	50%
10 Beads/cell (200 units/ml)	7398 ± 405	19%

Control - no beads or rHuGCSF added
Specific Activity = 0.002 units/bead

Table 17

Growth of DAI-E5 Cells on Covalent Versus Adsorbed rMuGMCSF Polystyrene Beads

Conditions	[^{3}H]-Thymidine Incorporation (DPM's)	% of the Control
Control	2562 ± 1316	0%
Soluble rMuGMCSF		
100 units/ml	28758 ± 2976	100%
Immobilized rMuGMCSF		
Covalent rMuGMCSF	12278 ± 1544	37%
Adsorbed rMuGMCSF	464 ± 75	-8%
Blank BSA beads	2124 ± 132	-2%

Bead concentration was 5×10^{6} Beads/well for the covalent and adsorbed beads.

Table 18

Growth of Human Lymphocytes Using Immobilized rHuILGF-I Polystyrene Beads

Conditions	Donor 1		Donor 2	
	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control
<u>Soluble rHuILGF-I</u>				
Control (nothing)	37075 ± 3280	0%	53481 ± 3074	0%
10 ng/ml	45408 ± 5013	100%	60590 ± 6405	81%
100 ng/ml	45209 ± 2856	98%	62271 ± 4712	100%
<u>Immobilized rHuILGF-I</u>				
52 ng	42089 ± 2027	72%	62353 ± 7067	101%
104 ng	41589 ± 4292	54%	60814 ± 4120	83%

Specific Activity = 0.00052 ng/Beads.

Table 19

Growth of Human Lymphocytes Using Immobilized rHuILGF-II Polystyrene Beads

Conditions	Donor 1: [^{3}H]-Thymidine Incorporation (DPM's)	Donor 1: % of the Control	Donor 2: [^{3}H]-Thymidine Incorporation (DPM's)	Donor 2: % of the Control
Soluble rHuILGF-II				
0	8444 ± 541	0%	19575 ± 1375	0%
100 ng/ml	12327 ± 906	75%	21857 ± 1320	21%
200 ng/ml	13621 ± 443	100%	30431 ± 1421	100%
Immobilized rHuILGF-II				
42 ng/ml	11963 ± 1256	68%	27550 ± 1161	23%
84 ng/ml	13588 ± 485	99%	32091 ± 3193	115%

Specific Activity: 0.00042 ng/Bead.

Table 20

Killing of Murine LM Cells by Immobilized Recombinant Human TNF-alpha Polystyrene Beads

Conditions	Experiment 1: Absorbance 590 nm (MTT uptake)	Experiment 2: Absorbance 590 nm (MTT uptake)	Experiment 3: Absorbance 590 nm (MTT uptake)	Experiment 3: [^{3}H]-Thymidine Incorporation (DPM's)
Soluble TNF-alpha				
Control	0.329 ± 0.045	0.358 ± 0.039	0.371 ± 0.083	204179 ± 11357
10 units/ml	0.350 ± 0.003	0.466 ± 0.108	-----	48283 ± 2472
100 units/ml	0.316 ± 0.039	0.436 ± 0.047	-----	43621 ± 12828
1000 units/ml	0.097 ± 0.011	0.270 ± 0.004	-----	11445 ± 13123
Immobilized TNF-alpha				
1 Bead/cell (384 units/ml)	0.205 ± 0.009	0.291 ± 0.024	0.305 ± 0.033	13174 ± 5310
5 Beads/cell (1920 units/ml)	0.165 ± 0.011	0.168 ± 0.004	0.029 ± 0.001	8794 ± 2748

Assay is a 72-hour killing assay that does not use Actinomycin D that inhibits DNA synthesis. MTT measures oxidation via the mitochondria to give insoluble blue crystals.
[^{3}H]-Thymidine uptake measures DNA synthesis.

Specific Activity = 0.038 units/bead

Table 21

Growth of Murine 3T3 Cells Using Immobilized FGFb Polystyrene Beads.

	Experiment 1		Experiment 2	
Growth Factor (concentration)	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control
Control	2682 ± 1124	0%	3627 ± 1402	0%
<u>Soluble FGFb</u>				
1 ng	4145 ± 584	82%	-----	
5 ng	3489 ± 441	34%	-----	
10 ng	2766 ± 810	5%	4276 ± 122	52%
50 ng	4474 ± 1115	100%	4880 ± 1261	100%
<u>Immobilized FGFb</u>				
18 ng	5546 ± 1230	160%	4172 ± 528	43%
36 ng	3184 ± 457	28%	2109 ± 162	0%
10% CS	38544 ± 6699		67235 ± 10758	

Control - no growth factors added, just DME medium plus 0.4% CS
1. 600 3T3 cells/well; 24 hour exposure to FGFb before isotope was added.
2. 2000 3T3 cells/well; 24 hour exposure to FGFb before isotope was added.
3. 2000 3T3 cells/well; 48 hour exposure to FGFb before isotope was added.

Specific activity = 0.000114 ng FGFb/bead

Table 21 (continued)

	Experiment 3		Experiment 4	
Growth Factor (concentration)	$[H^3]$-Thymidine Incorporation (DPM's)	% of the Control	$[H^3]$-Thymidine Incorporation (DPM's)	% of the Control
Control	4123 ± 1602	0%	4846 ± 1095	0%
Soluble FGFb				
1 ng	9747 ± 2295	59%	8114 ± 2522	54%
5 ng	11380 ± 4476	77%	10825 ± 1277	99%
10 ng	12275 ± 3496	86%	10871 ± 4152	100%
50 ng	13600 ± 2045	100%	9069 ± 3247	70%
Immobilized FGFb				
18 ng	19685 ± 3969	164%	6021 ± 507	20%
36 ng	10160 ± 912	64%	5224 ± 2109	6%
10% CS	80946 ± 23421		104907 ± 3450	

Table 22

Growth of NRK-49F Cells Using Immobilized TGF-beta-2 Polystyrene Beads

	Experiment 1		Experiment 2		Experiment 3	
Growth Factor (concentration)	[^{3}H]-Thymidine Incorporation (DPM's)	% of the Control	[^{3}H]-Thymidine Incorporation (DPM's)	% of the Control	[^{3}H]-Thymidine Incorporation (DPM's)	% of the Control
Control	14343 + 1859	0%	4725 ± 891	0%	6000 + 1776	0%
Soluble TGF-beta-2						
0.5 ng	16286 + 2808	38%	-----	0%	4129 + 3058	0%
1.0 ng	17704 + 3302	65%	4095 ± 201	0%	6041 + 791	0%
Immobilized TGF-beta-2						
7.0 ng	13036 + 2740	0%	-----		11260 + 245	25%
18 ng	15749 + 3669	27%	5533 ± 3557	30%	10301 + 1117	21%
36 ng	22663 + 2731	162%	6092 ± 1126	48%	8210 + 1976	11%
10% CS	19491 + 2001	100%	7520 ± 942	100%	26828 + 4923	100%

Control - no growth factors added, DME plus 0.2% CS.

Table 23

Killing of HeLa S3 Tumor Cells by Immobilized Recombinant Human INF-alpha-2a Polystyrene Beads

	48 Hour Exposure		72 Hour Exposure	
Conditions	[^{3}H]-Thymidine Incorporation (DPM'S)	% Killing	[^{3}H]-Thymidine Incorporation (DPM's)	% Killing
Soluble INF-alpha-2a				
Control	114547 ± 12672	0%	124630 ± 25451	0%
10 units/ml	56978 ± 10503	50%	73303 ± 27314	41%
100 units/ml	33000	71%	44062 ± 5708	65%
Immobilized INF-alpha-2a Beads				
24 Beads/cell (720 units/ml)	64411 ± 7375	44%	76756 ± 19220	38%
120 Beads/cell (7800 units/ml)	56237 ± 5652	51%	73735 ± 7160	41%

Specific Activity = 0.03 units/bead

Table 24

Growth of NRK-49F Cells Using Immobilized rHuEGF Polystyrene Beads

	Experiment 1 (24 hours)		Experiment 2 (48 hours)	
Conditions	[^{3}H]-Thymidine Incorporation (DPM's)	% of the Control	[^{3}H]-Thymidine Incorporation (DPM's)	% of the Control
<u>Soluble rHuEGF</u>				
Control	1355 ± 313	0%	945 ± 828	0%
50 ng/ml	8252 ± 1709	100%	1922 ± 661	100%
100 ng/ml	7252 ± 1002	86%	1714 ± 572	79%
<u>Immobilized rHuEGF</u>				
125 ng EGF	7352 ± 1070	87%	2275 ± 781	136%
625 ng EGF	7455 ± 1702	88%	1858 ± 239	93%
<u>Serum Controls</u>				
10% CS	16908 ± 1761		2985 ± 691	

Specific Activity = 0.000025 ng/bead

Table 25

Growth of Murine Swiss 3T3 Cells Using Immobilized PDGF Polystyrene Beads

Conditions	Experiment 1		Experiment 2	
	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control	$[^3H]$-Thymidine Incorporation (DPM's)	% of the Control
Soluble rHuPDGF				
Control	1015 ± 71	0%	749 ± 778	0%
1 ng/ml	3996 ± 2143	34%	1586 ± 584	27%
10 ng/ml	9795 ± 4128	100%	3847 ± 1559	100%
Immobilized rHuPDGF				
150 ng PDGF	2877 ± 719	21%	1278 ± 2390	17%
300 ng PDGF	9127 ± 903	92%	2575 ± 2390	59%
Serum Controls				
2% CS	5860 ± 4351		1634 ± 1144	
10% CS	65230 ± 17090		26235 ± 3477	

Specific Activity = 0.0001 ng/bead

Table 26

Growth of DA1-E5 Cells Using Immobilized Recombinant Human Erythropoietin (rHuEPO) Co-Bind™ Polystyrene Wells

Conditions	Experiment 1: $[^3H]$-Thymidine Incorporation (DPM's)	Experiment 1: % of the Control
Control	305 ± 104	0%
<u>Soluble rHuEPO</u>		
1 unit/ml	2363 ± 101	100%
<u>Immobilized rHuEPO</u>		
5 units/well	596 ± 465	14%
10 units/well	1013	34%
20 units/well	1879	76%

Table 26 (continued)

Conditions	Experiment 2	
	MTT Incorporation Absorbance at 590 nm	
Control	0.100	0%
Soluble rHuEPO		
1 unit/ml	0.280	100%
5 units/ml	0.272	96%
Immobilized rHuEPO		
50 units/well	0.274	97%
100 units/well	0.219	66%
200 units/well	0.295	108%

Table 27

Tumor Necrosis Factor Production (pg/ml)
By Monocytes Exposed to Immobilized and Soluble Gamma-Interferon

Concentration Gamma-Interferon	TNF Production (pg/ml) Immobilized	Soluble
control	2630	2630
1 unit		5950
10 units		8100
50 units	4150	
100 units	3600	12,500

WHAT IS CLAIMED IS:

1. An immobilized cytokine comprising a cytokine bound to a solid support, said immobilized cytokine having substantially the biological activity demonstrated by the free cytokine, and wherein said immobilized cytokine is reusable.

2. The immobilized cytokine of claim 1 wherein said cytokine is covalently bound to said solid support.

3. The immobilized cytokine of claim 2 wherein said cytokine is covalently bound to said solid support using a urethane, triazine ether, amine, or amide linkage.

4. The immobilized cytokine of claim 3 wherein said cytokine is covalently bound to said solid support using an amine or amide linkage.

5. The immobilized cytokine of claim 4 further including a linking arm wherein said cytokine is bound to said solid support by said linking arm and said linking arm comprises one or more linking groups selected from the group consisting of:

(a) diamines, having the general formula $NH_2-R^1-NH_2$, where R^1 is a C_2-C_{20} alkyl group;

(b) amino acids, having the general formula $NH_2-R^2-CO_2H$, where R^2 is a C_1-C_{20} alkyl group; and

(c) dialdehydes, having the general formula $OHC-R^3-CHO$, where R^3 is a C_1-C_{20} alkyl group.

6. The immobilized cytokine of claim 5 wherein said linking arm comprises one or more linking groups selectedfrom the group consisting of 6-aminocaproic acid, 1,6-diaminohexane, 1,12-diaminododecane, glutaraldehyde, and mixtures thereof.

7. The immobilized cytokine of claim 1 wherein said cytokine is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, tumor necrosis factor, gamma-interferon, alpha-interferon, beta-interferon, erythropoietin, granulocyte colony stimulating factor, murine granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, murine granulocyte-macrophage colony stimulating factor, insulin--like growth factor I, insulin-like growth factor II, transformation growth factor beta, epidermoid growth factor, platelet derived growth factor, and fibroblast growth factor basic.

8. The immobilized cytokine of claim 7 wherein said cytokine is selected from the group consisting of IL-2, GMCSF, GCSF, EPO, TNF, FGFb, TGFb, PDGF.

9. The immobilized cytokine of claim 8 wherein said cytokine is a polyethylene glycol-modified IL-2 or an ala-125 IL-2 analogue.

10. The immobilized cytokine of claim 1 wherein said solid support is nonporous.

11. The immobilized cytokine of claim 10 wherein said solid support is a substantially spherical bead having a diameter of about 0.5-500 μm.

12. The immobilized cytokine of claim 11 wherein said spherical bead has a diameter of about 1-75 μm.

13. The immobilized cytokine of claim 12 wherein said solid support is a staple fiber having a diameter of about 5-200 μm.

14. The immobilized cytokine of claim 10 wherein said support is selected from the group consisting of inorganic supports, including glass, quartz, ceramics, zeolites, metals, and metal oxides; polymeric materials including homopolymers, copolymers, or oligopolymers derived from monomeric units selected from the group consisting of styrene, divinylbenzene, ethylene, butadiene, acrylonitrile, acrylic acid, methacrylic acid, esters of acrylic and methacrylic acid, vinyl acetate, fluoroalkenes, acrylamide and methacrylamide; carbohydrate supports, including agarose, cross-linked agarose, dextran, inulin, hyaluronic acid, cellulose, cellulose derivatives, starch and starch derivatives; and insoluble protein materials, including gelatin, collagen and albumin.

15. The immobilized cytokine of claim 14 wherein said support comprises a homopolymer, copolymer, or oligopolymer derived from monomeric units selected from the group consisting of styrene, divinylbenzene, ethylene, butadiene, acrylonitrile, acrylic acid, methacrylic acid, esters of acrylic and methacrylic acid, vinyl acetate, fluoroalkenes, acrylamide and methacrylamide.

16. The immobilized cytokine of claim 10 wherein said support includes a functionalized surface having a plurality of functional groups selected from the group consisting of hydroxyl, amino, carboxyl, sulfhydryl, and halogen.

17. A method for the <u>in vitro</u> growth of a cytokine dependent cell line comprising inducing growth of said cell line by contacting it with an effective amount of a cytokine bound to a solid support.

18. The method of claim 17 wherein said dependent cell line is CTTL-2 and said cytokine is IL-2.

19. The method of claim 17 wherein said dependent cell line is AML-193 and said cytokine is selected from the group consisting of HuGMCSF, HuGCSF, and IL-3.

20. The method of claim 17 wherein said dependent cell line is Balb/c 3T3 and said cytokine is PDGF or FGF-beta.

21. The method of claim 17 wherein said dependent cell line is NRK-49F and said cytokine is TFG-beta or EGF.

22. The method of claim 17 wherein dependent cell line is DA1-E5 and said cytokine is erythropoietin.

23. A method for the in vitro growth of cellular blood components comprising inducing growth of said components by contacting said components with an effective amount of a cytokine bound to a solid support.

24. The method of claim 23 wherein said cellular blood components are human peripheral blood lymphocytes.

25. A method for the in vitro growth of effector cells selected from the group consisting of lymphokine activated killer cells, natural killer cells, tumor infiltrating lymphocytes, and cytotoxic T-cells comprising inducing growth of said cells by contacting said cells with an effective amount of a cytokine bound to a solid support.

26. A method for the in vivo stimulation of the natural killer or lymphokine activated killer cells in the immune system of a host comprising injecting an effective amount of a cytokine bound to a solid support.

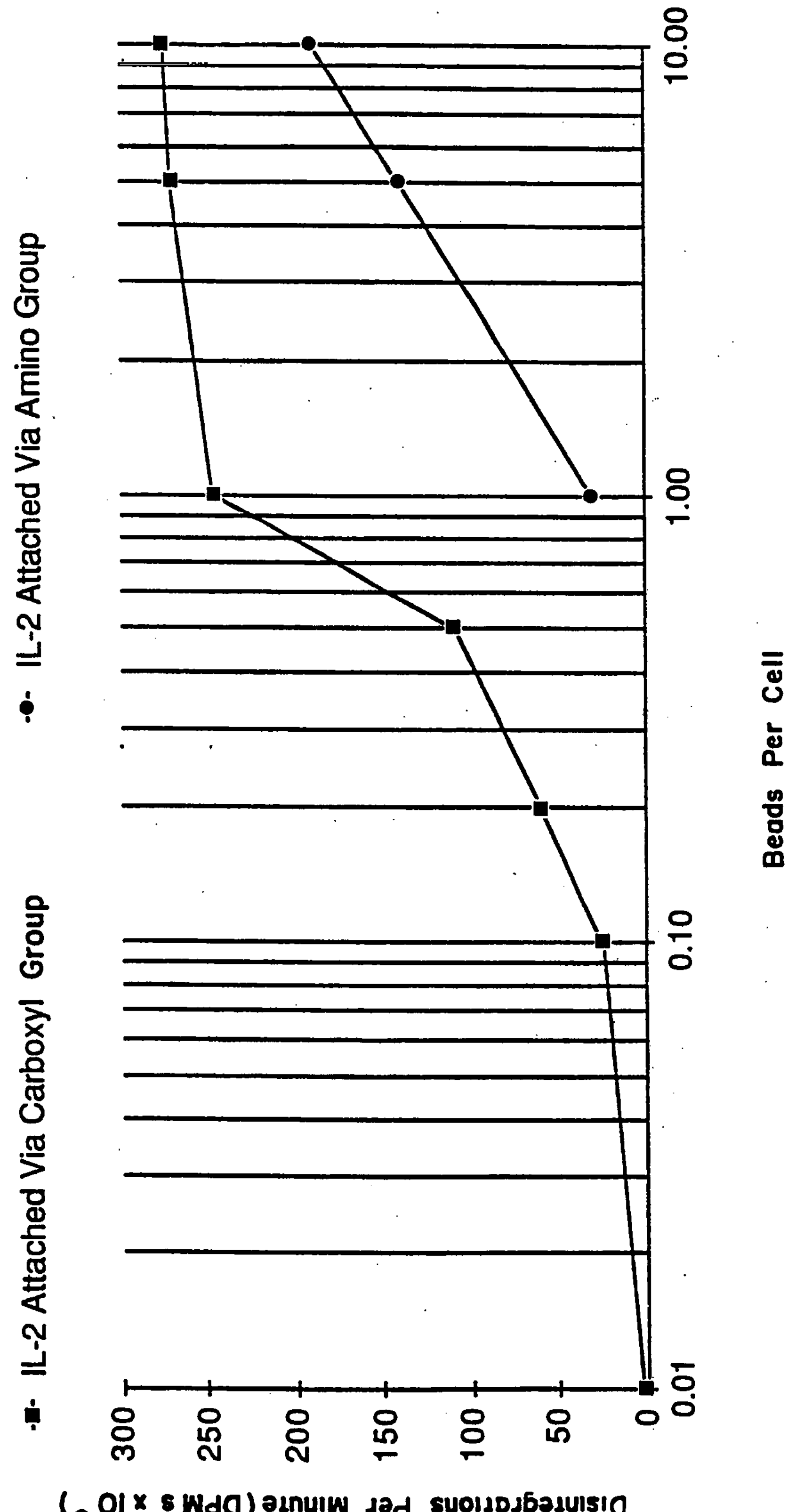
27. A method for the <u>in vivo</u> stimulation of hematopoietic cell growth of a host comprising injecting an effective amount of a cytokine bound to a solid support.

28. A method of claim 27 wherein said hematopoietic cells are granulocyte macrophages and said cytokine is GMCSF.

29. A method for the stabilization of a cytokine and substantially reducing proteolytic degregation <u>in vivo</u>, comprising attaching said cytokine to a solid support prior to introduction into the host.

30. A method for preventing the systemic absorption of cytokines, and the toxicity caused by the absorption of cytokines, comprising attaching said cytokine to a solid support, prior to introduction into the host.

FIG. 1



-■- IL-2 Attached Via Carboxyl Group
-●- IL-2 Attached Via Amino Group
Disintegrations Per Minute (DPM's x 10^{-3})
300
250
200
150
100
50
0
0.01
0.10
1.00
10.00
Beads Per Cell

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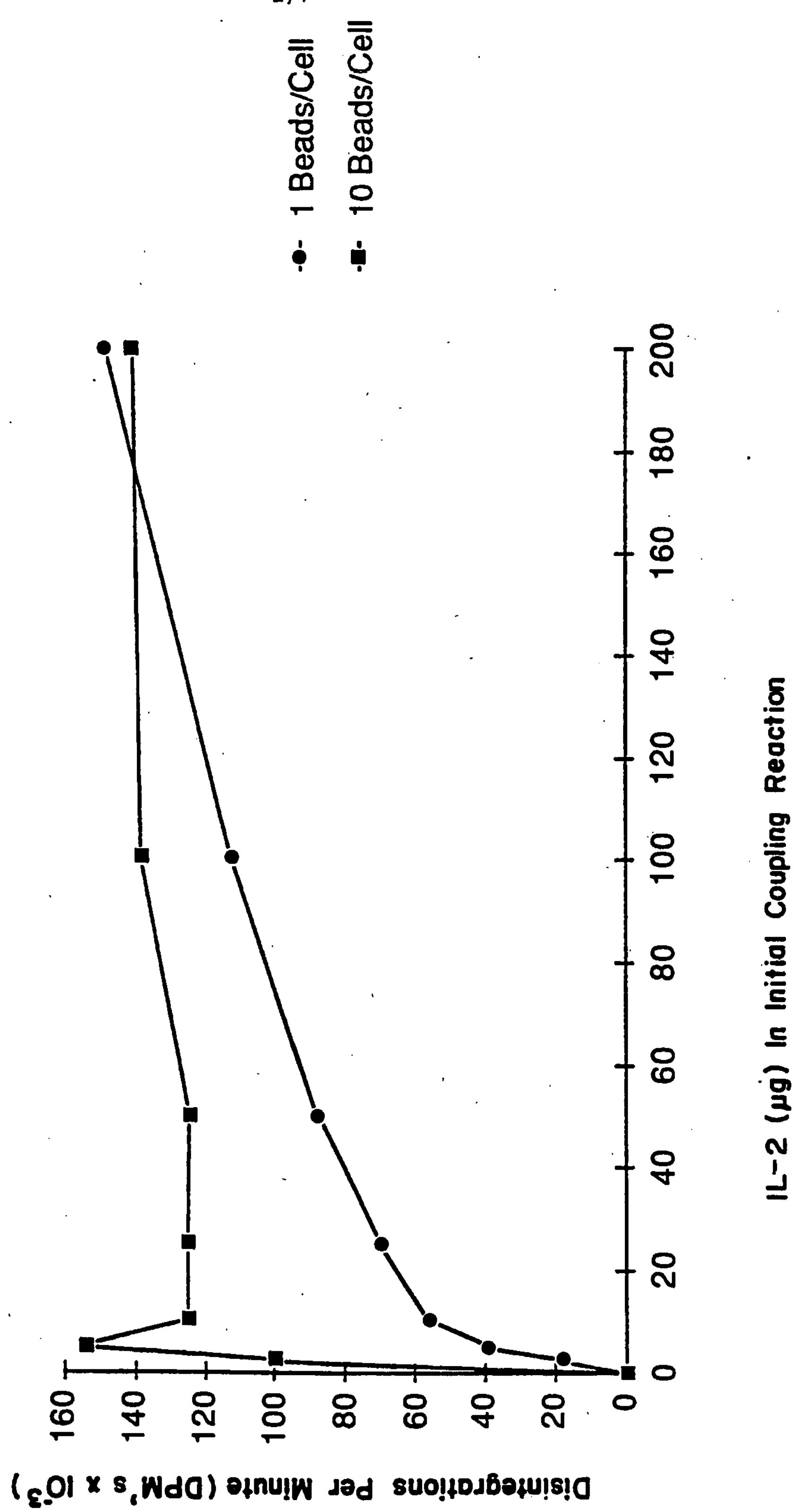
FIG. 2



1 Beads/Cell
10 Beads/Cell
Disintegrations Per Minute (DPM's x 10^-3)
0
20
40
60
80
100
120
140
160
180
200
IL-2 (µg) In Initial Coupling Reaction

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FIG. 3

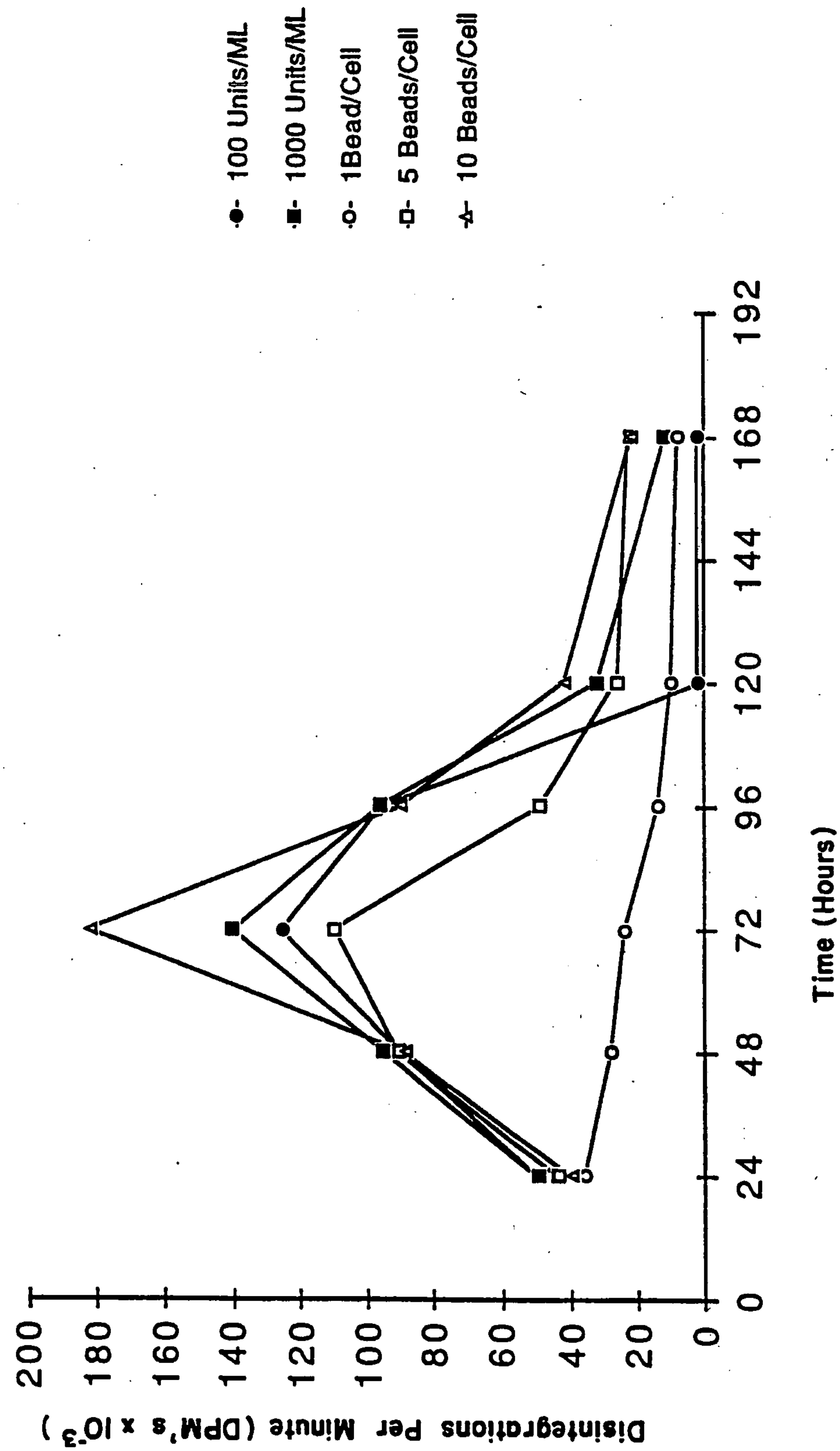
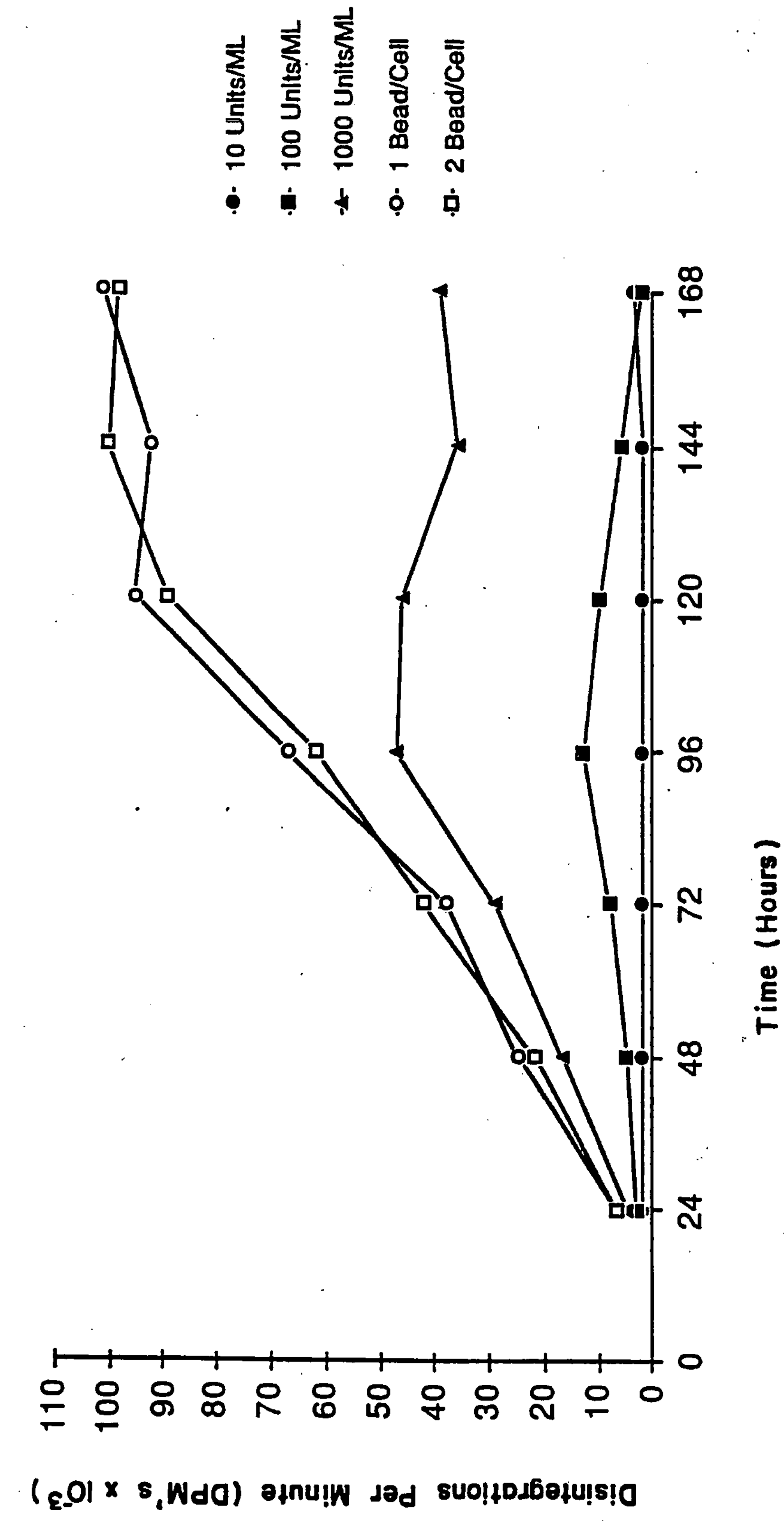

100 Units/ML
1000 Units/ML
1Bead/Cell
5 Beads/Cell
10 Beads/Cell
Disintegrations Per Minute (DPM's x 10^-3)
0
20
40
60
80
100
120
140
160
180
200
Time (Hours)
0
24
48
72
96
120
144
168
192

FIG. 4


10 Units/ML
100 Units/ML
1000 Units/ML
1 Bead/Cell
2 Bead/Cell
Disintegrations Per Minute (DPM's x 10^3)
Time (Hours)
0 10 20 30 40 50 60 70 80 90 100 110
0 24 48 72 96 120 144 168

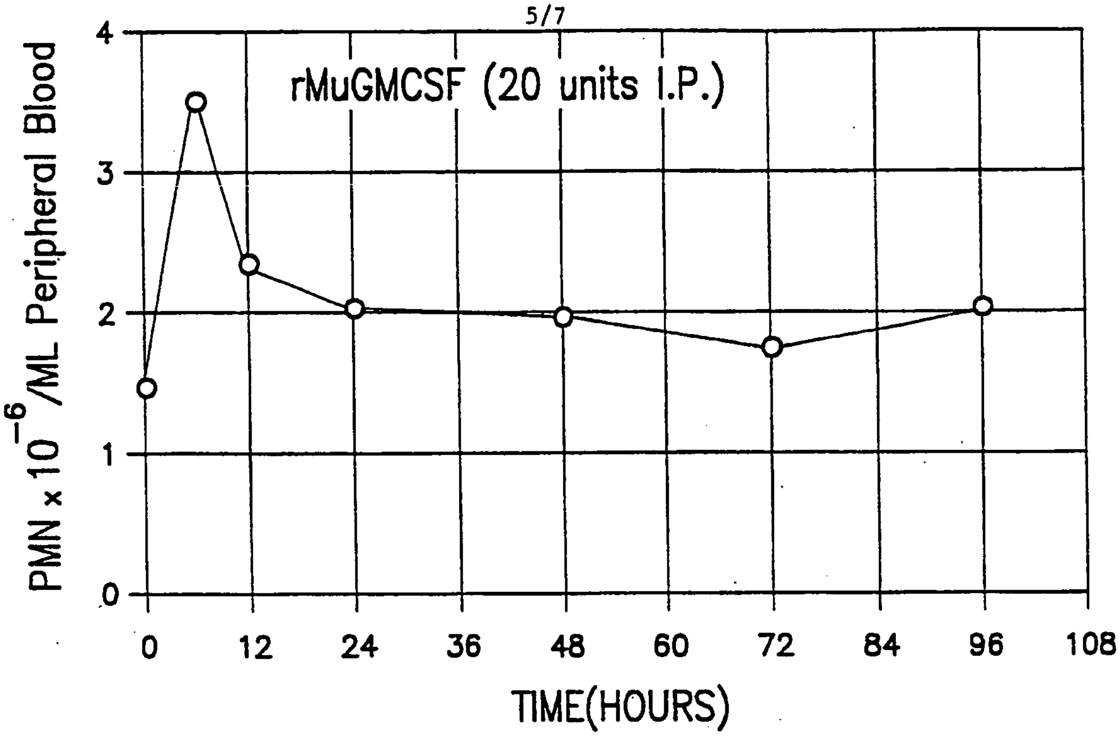

rMuGMCSF (20 units I.P.)
PMN x 10^{-6}/ML Peripheral Blood
4
3
2
1
0
0
12
24
36
48
60
72
84
96
108
TIME(HOURS)

FIG. 5A

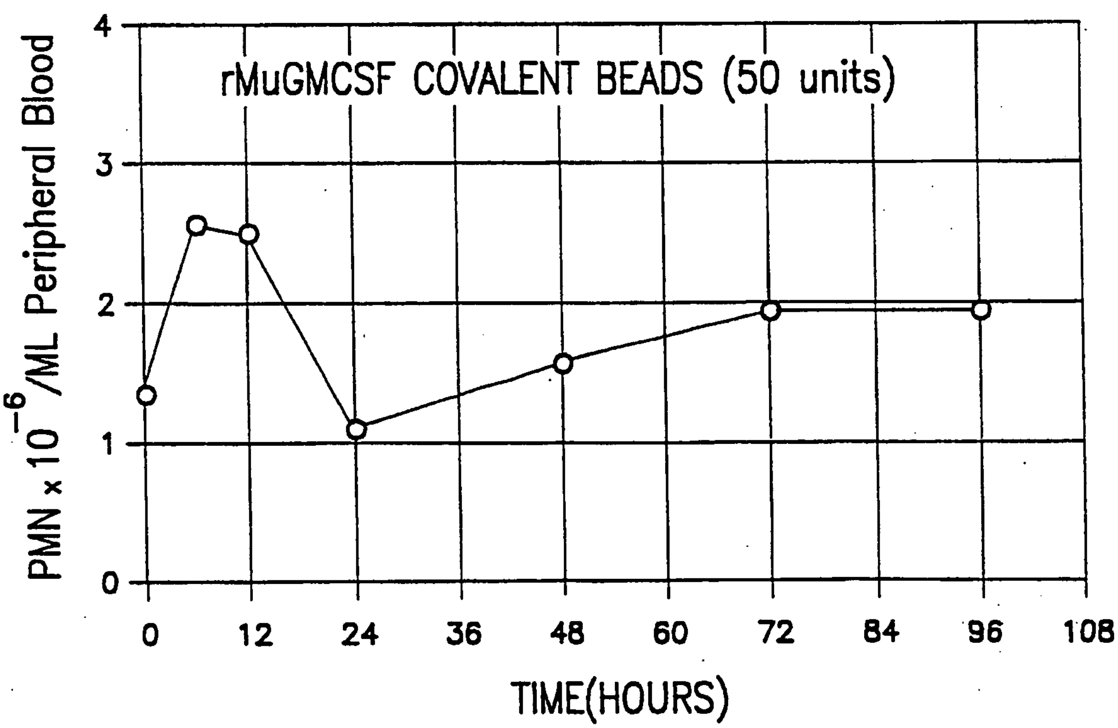

rMuGMCSF COVALENT BEADS (50 units)
PMN x 10^{-6}/ML Peripheral Blood
4
3
2
1
0
0
12
24
36
48
60
72
84
96
108
TIME(HOURS)

FIG. 5B

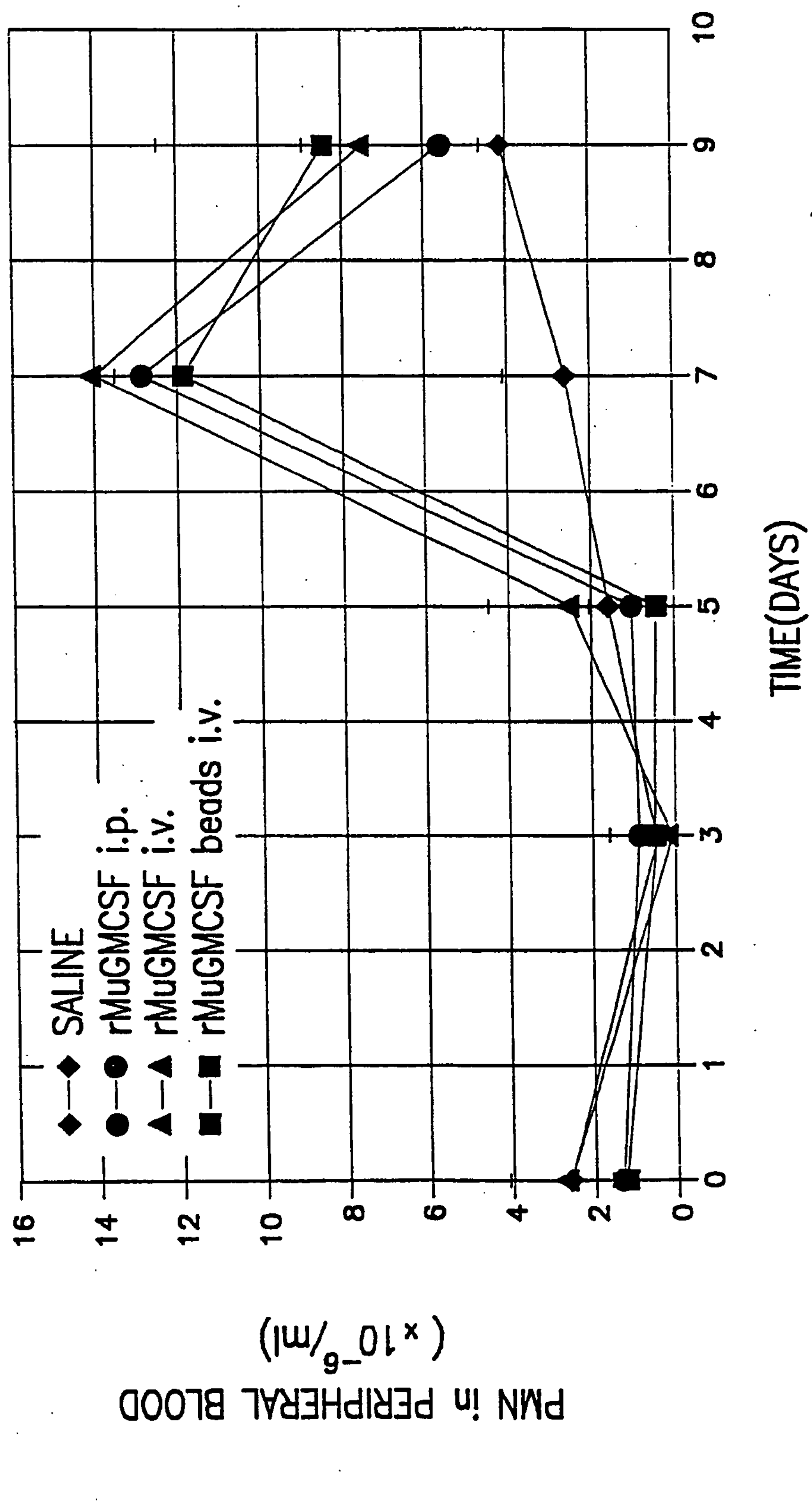

SALINE
rMuGMCSF i.p.
rMuGMCSF i.v.
rMuGMCSF beads i.v.
PMN in PERIPHERAL BLOOD
(x10^-6/ml)
TIME(DAYS)
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5
6
7
8
9
10

FIG. 6

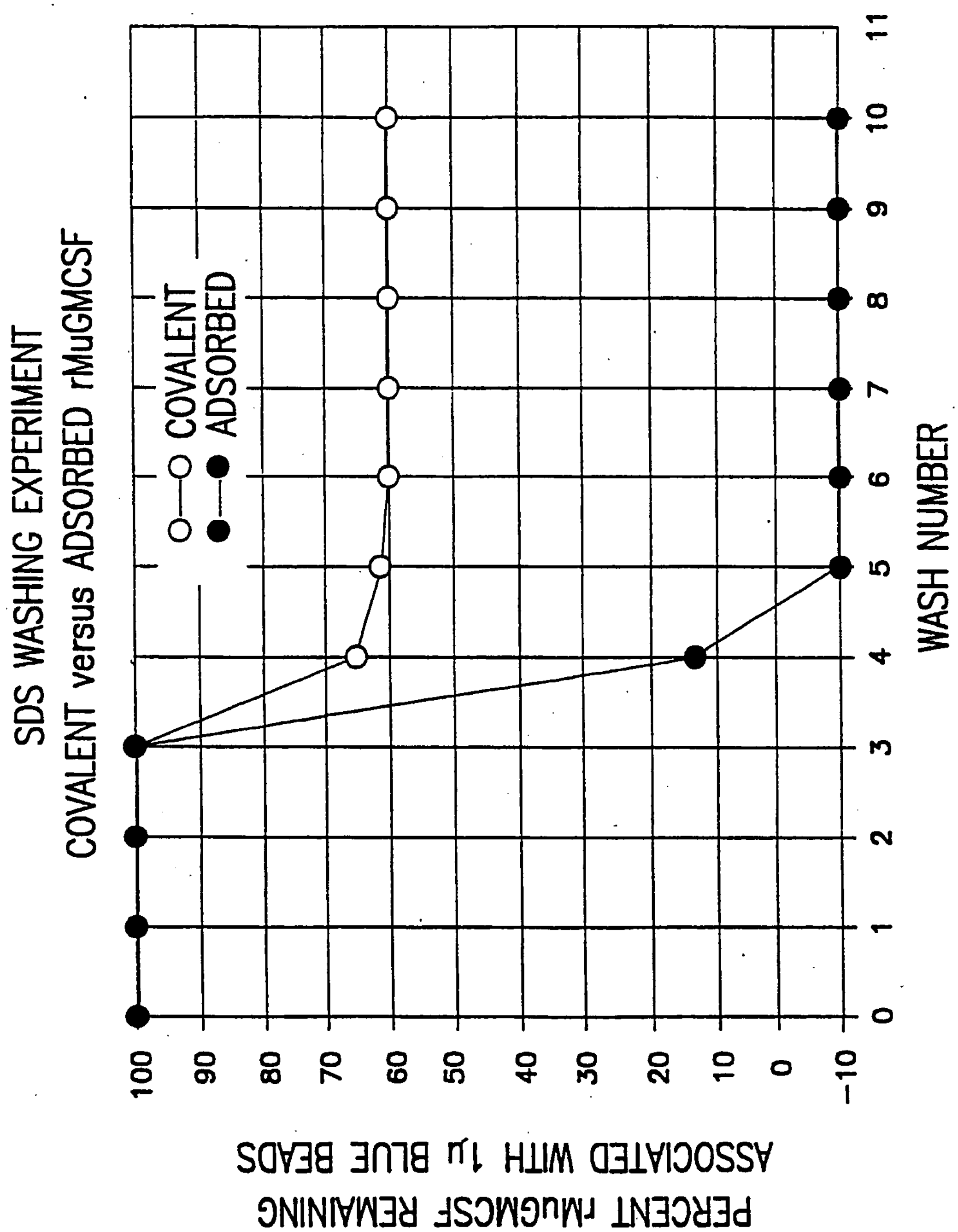

SDS WASHING EXPERIMENT
COVALENT versus ADSORBED rMuGMCSF
COVALENT
ADSORBED
PERCENT rMuGMCSF REMAINING
ASSOCIATED WITH 1μ BLUE BEADS
100
90
80
70
60
50
40
30
20
10
0
−10
0
1
2
3
4
5
6
7
8
9
10
11
WASH NUMBER

FIG. 7

ITERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01031

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/00,37/10; C07K17/06,17/08,17/10,17/14;C12N 5/00;(con't)
U.S. CL.: 514/2,8; 530/811,813,815,816; 435/240.2;424/85.1 (con't)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/2,8;530/810,811,812,813,814,815,816; 435/174,177,178,180,181,240.1,240.2,240.21, 240.51;424/78,85.1,85.2,85.4,85.5,85.6,85.7

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
X / Y	JP,A, 61-53,300 (KATO et al). 17 MARCH 1986, See entire document.	1-10,14-16 / 11-13,17-30
X / Y	US,A, 4,609,546 (HIRATANI) 02 SEPTEMBER 1986, See entire document	1-4,7-10,14-16 / 5,6,11-13,17-30
Y	US,A, 3,639,213 (GINGER et al) 01 FEBRUARY 1972, See entire document	11-13
Y	US,A, 4,240,662 (RECKEL et al) 20 FEBRUARY 1979, See entire document	11-13
Y	US, A, 4,764,466 (SUYAMA et al) 16 AUGUST 1988, See entire document	11-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

21 MAY 1990

Date of Mailing of this International Search Report

11 JUL 1990

International Searching Authority

ISA/US

Signature of Authorized Officer

DAVID M. MATT

Form PCT/ISA/210 (second sheet) (Rev.11-87)

CON'T OF CLASSIFICATION OF SUBJECT MATTER

IPC(5): A61K 45/05, 37/66
U.S. CL: 424/85.2,85.4,85.5,85.6,85.7